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Amperometric Enzyme-based Biosensors for Lowering the Interferences

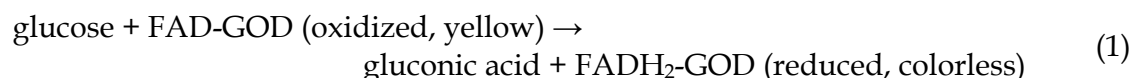
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1. Introduction

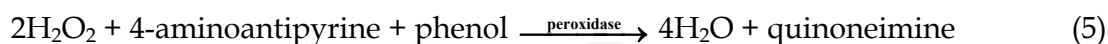
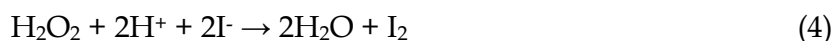
1.1 Glucose, enzymes and mediators

Glucose becomes more and more important and popular research topics for medicine and biochemistry that monitoring biomarkers of chronic diseases, such as glucose to diabetes, bilirubin to jaundice and creatinine to kidney disease. Among many biomarkers, glucose is a common and an important biological species of human blood, found out normally in the range of about 4~8 mM. According to statistical information system of World Health Organization (WHO), the number of people with diabetes is estimated more than 180 million worldwide and it is likely to more than double by 2030. Besides, it is also estimated that 9% of all deaths worldwide are due to diabetes. Most notably, diabetes deaths are projected to increase by over 80% in upper-middle income countries between 2006 and 2015. Therefore, it is necessary to develop an efficient glucose biosensor for monitoring the glucose level of diabetics.

Glucose is an attractive target, because it is not only an important biomarker for diabetes but also a kind of fuel for biofuel cells. In other words, the glucose biosensor can work for detecting the glucose level and for the anode of the biofuel cell. The biofuel cells were intended to power cardiac assist devices, such as artificial hearts or cardiac pacemakers (Rao & Richter, 1974; Rao et al., 1974). For getting a good specific property, enzymes are widely applied as recognized molecules. Two kinds of enzymes with different redox potentials and electron transfer pathways are usually used to catalyze the glucose. One is the glucose oxidase (GOD) (Franke & Deffner, 1939) from *Aspergillus niger* and the other is glucose dehydrogenases (GDH) from *Acinetobacter calcoaceticus*. For GOD catalyst, the cofactor is flavin adenine dinucleotide (FAD) with a strong bond to apo-GOD, but the cofactor can be nicotinamide adenine dinucleotide (NAD) (Boguslavsky et al., 1995), FAD (Tsujimura et al., 2006) and pyrrole quinoline quinone (PQQ) (Duine et al., 1979) for GDH. As an example, the FAD-GOD was selected in this chapter as the recognized molecule. The FAD-GOD has an apparent formal redox potential of -0.048 V vs. standard hydrogen electrode (SHE) (Kulys et al., 2006) and it has a catalyzed rate of 5×10^3 glucose molecules per second (Willner et al., 2007a). The series catalytic mechanisms in a solution with oxygen are shown in Eqs. (1) and (2) (Warburg & Christian, 1932).



Source: Intelligent and Biosensors, Book edited by: Vernon S. Somerset,
ISBN 978-953-7619-58-9, pp. 386, January 2010, INTECH, Croatia, downloaded from SCIYO.COM



The glucose concentrations can be determined indirectly by the consumption of O_2 or the further reaction of H_2O_2 . For example, the amperometric current can be collected by the oxidation of H_2O_2 directly (shown in Eq. (3)) (Chaubey & Malhotra, 2002), the reaction of H_2O_2 and I^- gave a I^-/I_2 potentiometry (shown in Eq. (4)) (Malmstadt & Pardue, 1961), and a spectrum change of the red dye (quinoneimine) was observed based on the reaction of Eq. (5) (Nien et al., 2008). However, the above sensing signals are sensitive to ambient oxygen concentration by any detection methods, so mediators are added into the system as shown by Eq. (6) instead of Eq. (2). The electron transfer from the redox center of FADH_2 to an electrode is very sluggish and hard, because the FADH_2 is embedded inside GOD by glycoprotein at a distance of about 1.3-1.5 nm (Hecht et al., 1993). The mediators not only facilitate the electron transfer from FADH_2 to electrodes but also lower the sensing potential, so the choice of the mediator is very important to the sensing performance. Cyclic voltammogram is a good electro-analytical method to obtain the properties of mediators and to find suitable mediators (Gilmartin & Hart, 1995; Nakaminami et al., 1997). The most used mediators for GOD with their formal potentials *vs.* standard calomel electrode (SCE) are partially listed in Table 1 (Chaubey & Malhotra, 2002). Generally speaking, the mediators can be classified into three kinds, including organic, inorganic and metal-organic (Heller & Feldman, 2008). In the organic mediators, methylene blue (Karyakin et al., 1993; Willner et al., 2007a; Willner et al., 2007b), quinone and its derivatives (Battaglini et al., 1994; Bourdillon et al., 1986; Cenas et al., 1983; Cosnier et al., 1998; Williams et al., 1970) have been studied for a long time. In the second kind, the main inorganic mediators are the hexacyano-complexes of iron (Dubinin et al., 1991; Jaffari & Turner, 1997; Shulga et al., 1994), cobalt and ruthenium, especially $\text{Fe}(\text{CN})_6^{4-}$ is widely used in commercial glucose strips. In the final category, the metal-organic mediators cover ferrocene (Hendry et al., 1993; Luong et al., 1994), ferrocenemethanol (Bourdillon et al., 1995; Yang et al., 2003; Zhang et al., 2005; Zhang et al., 2006b; Zhao & Wittstock, 2005), ferrocenecarboxylic acid (Chen et al., 2002; Kohma et al., 2007; Tian & Zhu, 2002), Os-complex (Mano et al., 2005; Mao et al., 2003; Zakeeruddin et al., 1992) and so on. Besides, Wang et al. reported that the multi-walled carbon nanotubes

Mediators	Formal potentials (mV <i>vs.</i> SCE)
1,1-dimethyl ferrocene	100
ferrocene	165
ferrocene carboxylic acid	275
hydroxyl methyl ferrocene	185
benzoquinone	39
$[\text{Fe}(\text{CN})_6]^{4-}$	180

Table 1. A partial list of the commonly used mediators for GOD.

(MWCNTs) can disturb the secondary structure of GOD and get close to its redox center to pass the electron directly without mediators (Wang et al., 2009).

1.2 The immobilization of enzymes and mediators

Among various detection methods, the amperometric enzyme-based biosensor probably is the best choice for biochemical analysis due to its good selectivity, high sensitivity, rapid response, convenient measurement, miniature size, and reproducible results (Hamdi et al., 2006). In order to reuse the expensive recognized biomolecules, the enzyme has to be immobilized on the electrode. In 1972, the company of Yellow Spring Instrument in America manufactured the first commercial glucose biosensor according to the prototype of the enzyme-immobilized electrode reported by Clark and Lyons (Clark & Lyons, 1962). In the following decades, the immobilization of enzyme became a key issue in developing the enzyme-based biosensor. Generally speaking, the immobilized methods (Cunningham, 1998) of enzyme include adsorption (Chu et al., 2007; Ekanayake et al., 2007), entrapment (Ngounou et al., 2007; Seo et al., 2007), cross-linking (Akyilmaz & Yorganci, 2007) and covalent bonding (Lin et al., 2007a; Seo et al., 2007). For adsorption, the enzyme was attached on the electrode by the attractive force of hydrogen bonds or opposite charges, such as nylon (Gamati et al., 1991) and ion exchange resin (Zhujun & Seitz, 1986), but it did not form a good adhesive force between biomolecules and a transducer. The enzyme also can be entrapped in a matrix, such as sol-gel (Lin et al., 2007b), Nafion® (Bogdanovskaya et al., 1997) and a conducting polymer (Brahim et al., 2001; Singh et al., 2004). The cross-linking and covalent bonding methods must be carried out by specific functional groups to link together, such as $-NH_2$ and $-COOH$ groups (Battaglini et al., 2000; Tamiya et al., 1990) or cross-linking agents (Tamiya et al., 1990). In addition, there are other methods used to immobilize the enzyme on the electrodes by thermal inkjet printing (Setti et al., 2005) or by enzyme-linked-immunosorbent-assay (ELISA) (Sehr et al., 2001). Among all methods, entrapment is considered to be one of the most attractive and popular methods to grasp the biomolecules. The electrochemical devices made by different conducting polymers entrapping recognized biomolecules have been reported extensively (Habermuller & Schuhmann, 1998; Rahman et al., 2004; Selampinar et al., 1997), because their major advantages (Cosnier, 1999) are that polymer film can be polymerized with immobilizing enzyme in one step, and the film thickness can be controlled easily by adjusting capacity. The most common polymers used as matrixes to entrap enzyme are polyaniline (Borole et al., 2004), polythiophene, polypyrrole and its derivatives (Trojanowicz et al., 1995). In addition to enzymes, the mediators should be immobilized in the same matrix for biosensors or biofuel cells. However, the immobilization of mediators is more difficult than that of enzyme, because the mediators usually suffer from the leakage of small molecules, water-insoluble. In the literatures reported, the covalent method is a more effective way to stabilize the mediators on the electrode. For example, the mediators were linked on the MWCNTs (Qiu et al., 2009), the polymer matrix (Himuro et al., 2009) or even the enzyme directly (Wu et al., 2008). Moreover, the mediator was linked with the electrode and the redox center of enzyme for increasing the efficiency of electron transfer from enzymes to the external circuit through mediators (Zayats et al., 2008). Qiu et al. (Qiu et al., 2007) proposed that the small molecules, mediators, were linked on the large molecules, $Fe_3O_4@SiO_2$ nanoparticles, and afterward the matrix entrapped the enzyme and the nanoparticles at the same time.

1.3 Challenges

In the aspect of clinical diagnosis, the selectivity is the most major concern. For the amperometric enzyme-based biosensor which is the subject of this chapter, the challenges are how to lower the interference signals and get a precise value of glucose level in real samples. There are many oxidation-favored species in whole blood resulting in extra amperometric sensing signals, and it is a major problem of selectivity especially for electrochemical sensing.

In order to eliminate this factor, a cationic exchange membrane (Nafion®) was the most common and easy way to put outside the electrode and this can prevent the negatively-charged interfering species, such as ascorbic acid, from reaching the surface of the electrode (Chen et al., 2009; Mailley et al., 2000; Wu et al., 2002; Zhang et al., 1994). But, the Nafion® film raised the resistance of ion-transport, in which, the response time may be increased. Another way to eliminate the interference effect is to set a pre-reaction zone on the upstream of the major sensing section. For example, L-ascorbate oxidase was immobilized in the front section of the channel (Kurita et al., 2002) to catalyze the ascorbic acid, but the other interferences passing to the electrode may still result in noises. Besides, for an electrochemical system, a new way to decrease the interferences is by means of applying different potentials for targets and interfered species. In previous study (Yuan et al., 2005), two different potentials were applied at the two working electrodes attached to scanning electrochemical microscopy (SECM) system where one was at low potential (0.5 V) on the substrate of glucose oxidase modified electrode and the other was at high potential (0.7 V) on the tip of bare platinum. Therefore, the oxidation-favored species reacted on the substrate electrode at low potential and the glucose can be catalyzed by the enzyme-modified electrode to produce hydrogen peroxide. Afterward, the H₂O₂ was oxidized again on the tip of the electrode when the gap between tip and substrate electrodes was small (11 μm). Based on the similar idea, Jia et al. (Jia et al., 2008; Jia et al., 2007) proposed the probe-in-tube microdevice for eliminating the interference by the tube and detecting the target by the enzyme immobilized probe.

2. Reviews and motivations

In the past decade, the technique of Micro-Electro-Mechanical-Systems (MEMS) has become more and more popular for fabricating sensor chips. Due to the recent development in biotechnology, bio-MEMS is widely incorporated into the microfluidic devices in biosensors with the recognized biomolecules. The sensing chips integrate the steps of sampling, reaction, separation and detection on a chip (Richter et al., 2002). Nevertheless, they miniaturize the size and have the properties of fast response, less sample and low cost (Auroux et al., 2002) and this kind of sensing chip is also called Lab-on-a-chip. For example, the biosensors based on the field effect transistor (FET) made by MEMS immobilize anti-PSA on the carbon nanotubes (CNTs) (Kojima et al., 2005), liquid-chromatography-based biochip detects peptide mixture (Xie et al., 2005), and the biochip combines PCR-based DNA amplification and electrochemical detection (Lee et al., 2003) have been reported. Other few examples include antibody-based chips for determining protein isoform (Loonberg & Carlsson, 2006), liquid-chromatography-based chips for detecting peptide mixture (Xie et al., 2005), and electrophoresis-based chips for sensing catechol and dopamine (Schoning et al., 2005). Moreover, there are many choices for the materials of the microchannel, such as poly(dimethylsiloxane) (PDMS) (McDonald et al., 2000), poly(methyl-methacrylate)

(PMMA) (Ford et al., 1998) and polycarbonate (PC) (Liu et al., 2001) ... etc. Among this, PDMS offers many advantages, including outstanding elasticity, pervious to light, good bio-compatible, good mechanical stability and convenient to be fabricated, and it can be used not only for the channel stamp but also for the gas-pump (Unger et al., 2000) and gas-valve (Hosokawa & Maeda, 2000). The PDMS stamp of channel is prepared with an air section between two layers. By filling and releasing gas to the air sections inside, the lower PDMS layer of channel can close and open the fluidic way in the micro-channel as a valve. Further, the fluid can be moved by gas-pump which is operated by three or more air sections in series filled and released continuously.

In the aspect of the electrodes on the MEMS-based biochip, the interdigitated ultramicroelectrode arrays (IDUAs) are usually used as they offer several advantages, including low ohmic drop (iR drop), high response time, enhanced sensitivity and increased signal-to-noise ratio (S/N ratio). The redox cycle of the species in IDUA was proposed by Bard et al. in 1986 (Bard et al., 1986). Further applications using IDUA (Fiaccabrino et al., 1998; Sheppard et al., 1996) and the search for the parameters of IDUA (Min & Baeumner, 2004) also have been reported. Additionally, there is another new way to increase the sensitivity up to 50 times by nanopores (Muller et al., 2007). The metal-insulator-metal electrode was created with many porous caves formed by nanoparticles then etched these caves to form cylindrical holes by plasma in CF₄, so the porous electrode can achieve electrochemical redox cycles in each hole vertically.

Yamato et al. (Yamato et al., 1995), firstly demonstrated that a polythiophene derivative, poly(3,4-ethylenedioxythiophene) (PEDOT), has a better long-term electrochemical stability than that of polypyrrole, and the good stability of the PEDOT was also confirmed by other researchers (Kros et al., 2005; Lerch et al., 1998). They all show that the PEDOT is a suitable material for electrochemical biosensor, so it was mainly acted as the matrix to entrap the enzyme in the studies. According to the literatures, PEDOT film not only can entrap glucose oxidase (Fabiano et al., 2002; Nien et al., 2006) or polyphenol oxidase (Vedrine et al., 2003) to fabricate a specific biosensor, but also detect single strand DNA directly (Krishnamoorthy et al., 2004).

In this chapter, two systems were reviewed to cover a good interference-independent glucose biosensor. One (system A) was designed that a three-electrode pattern was fabricated on the glass substrate by combining the technique of MEMS and covering a microchannel by the PDMS to form a sensing chip, thus the biochip worked in a flow system with the advantages of miniature, reuse, less injecting sample and continuous operation. Based on the electrochemical method, the recognized biomolecules, glucose oxidase, was immobilized by the conducting polymer, PEDOT, on the working electrode of the biochip for determining the glucose concentration. The enzyme-immobilized working electrode directly senses the catalyzed product, H₂O₂, according to Eqs. (1)-(3). Besides, a second working electrode of bare platinum, which is located in the near front of the first enzyme-immobilized working electrode, is designed for eliminating the oxidation-favored interferences near the surface of electrode before the fluid in microchannel reaching the enzyme-immobilized working electrode by applying the same potential. The schematic of the whole microfluidic sensing system, both the lateral and vertical views, is illustrated in Fig. 1.

The other (system B) is that the all-in-one electrodes, which co-immobilize with the enzymes and the mediators based on layer by layer structure shown in Fig. 2. The first layer, the

carbon paste which was coated on the flexible substrate of stainless steel (ssteel), was acted as an adsorbent layer for the mediator, p-benzoquinone (BZQ), by the hydrophobic force. The BZQ and GOD were drop-coated on the electrode in order and the entrapped matrix, PEDOT, was electropolymerized on the outer layer to prevent the leakage of mediators and enzymes. The all-in-one electrode has the advantages of flexible, workable in oxygen-independent solution, convenient, reusable, lower sensing potential and lower interference effect.

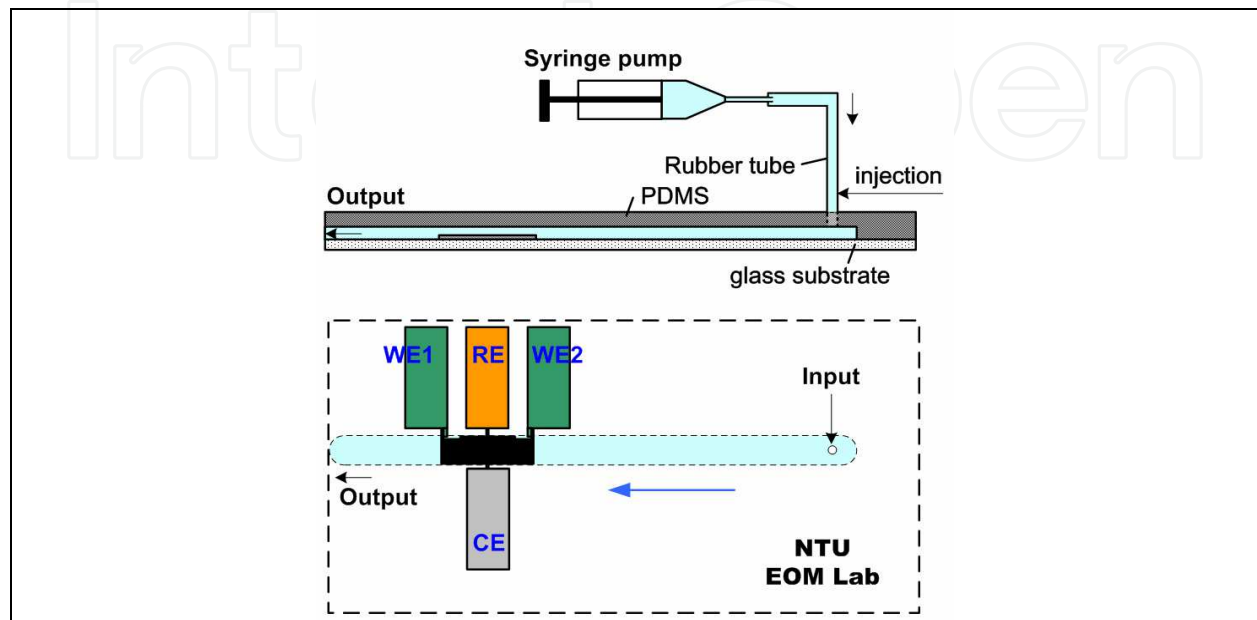


Fig. 1. The schematic of the whole system in operation for system A. (Nien et al., 2008)

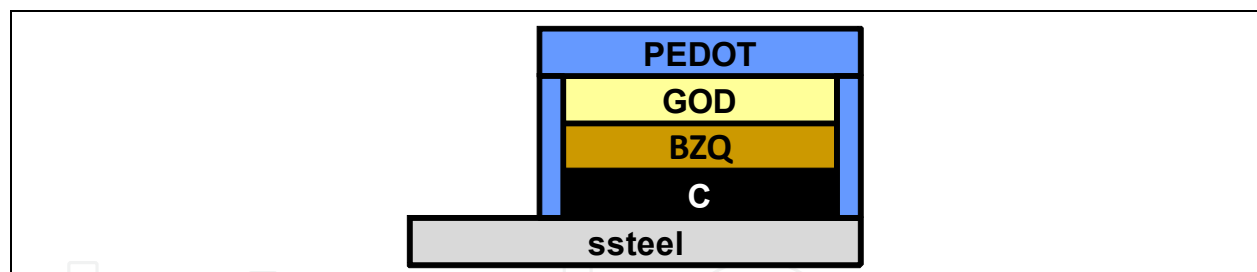


Fig. 2. The schematic of the layer by layer structure for system B.

3. Experimental

3.1 Chemicals and instruments

The target (or fuel), D-(+)-glucose, and the interferences, ascorbic acid (AA) (> 99%), uric acid (UA) (> 99%), dopamine hydrochloride (DA) and acetaminophen (AP) (> 99%), were purchased from Sigma. For the enzymes, glucose oxidase (GOD) (EC 1, 1, 3, 4) type VII-S from *Aspergillus niger*, and laccase (Lac) (EC 1, 10, 3, 2) from *Trametes versicolor* were purchased from Sigma and Fluka, respectively. For the mediators, p-benzoquinone (~ 98%, reagent grade) 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt were purchased from Aldrich and Sigma, respectively. The monomer, 3,4-ethylenedioxythiophene (EDOT), surfactant, polyethylene glycol (PEG, MW=20,000) and bacteriostat, sodium azide (>99.5%) were purchased from Aldrich, Merck and Sigma,

respectively. The phosphate buffer containing monosodium phosphate monohydrate, disodium phosphate heptahydrate and potassium chloride (99.0~100.5%), were all purchased from Sigma. Besides, in system A, the positive photoresist, FH-6400, and the developer, FHD-5, were purchased from Fujifilm. The pre-polymer PDMS (Sylgard 184) and curing agent were from Dow Corning. The photolithographic equipments in clean-room are following: UV mask aligner (EVG 620) and inductively coupled plasma-reactive ion etching (ICP-RIE). The solution in the channel was pumped by a syringe pump (KdScientific, model 100). In system B, the flexible substrate was stainless steel SUS 301 and the membrane for biofuel cell was Nafion® 117 (thickness is 0.007 in). The de-ionized water (DIW) was used throughout the experiments. All electrochemical experiments, including CV and amperometric measurements were performed with a potentiostat/galvanostat (CHI 440 and CHI 900).

3.2 Fabrication of system A

First, the film mask with a resolution of 10,000 dpi was made by Taiwan Kong King Company according to the self-designed electrode shown in Fig. 3. All of the following steps were done by silicon planar technology in a clean-room environment. The glass wafer with a diameter of 4 in and a thickness of 1 mm was cleaned by acetone, water and N₂-purge orderly. For enhancing the adhesive force between photoresist and glass wafer, the wafer was coated with hexamethyldisilazane (HMDS) in advance by vapor priming. Then the glass wafer was covered with chemical positive photoresist (FH-6400) by spin-coating at 1,500 rpm for 30 s and hardened at 90 °C for 90 s on a hot plate. After soft baking, the wafer was selectively exposed through a UV mask aligner to UV light (12 s, 10 mJ/cm²) with the first mask, and removed photoresist in the developer soup (FHD-5) for 12 s to form the pattern of the reference electrode. For the reference electrode, the metal layers of Cr, Au and Ag were deposited by sputtering in order, and the thicknesses of those are about 30, 90 and 360 nm, respectively. The layers of Cr and Au are served as buffer layers to enhance the adhesive force of silver on glass. The unnecessary metal layers were lifted off completely in the acetone solution by ultrasonic method. In the same process, the patterns of working and counter electrodes with the metal layers, 30 nm Cr and 100 nm Pt, were fabricated by the second mask. The working and counter electrodes are the designation of IDA with the same width (50 μm) of fingers and gaps, as shown in the insert of Fig. 3. The real geometric surface areas of working, counter, and reference electrode are 4.5, 9.75 and 1.3 mm² respectively.

The channel stamp was made by PDMS according to the mother mold of the silicon wafer. First, the silicon substrates were washed with acetone and sulfuric acid to remove any organic contaminants. All the substrates were then dried under a N₂ stream and used immediately after cleaning. Silicon wafers were coated with a 2 μm thick positive photoresist (FH-6400) by using a spin coater. They were pre-baked on a hot plate with a temperature of 90 °C for 90 s. The light exposure was followed for 12 s and developed for 12 s. Finally, silicon wafer was dry-etched by ICP-RIE for 100 μm deep and the patterns were transferred to the silicon mold. A fully mixed viscous precursor of PDMS and curing agent in the ratio of 10:1, was poured into the silicon master, pumped in a vacuum for a period of time to remove all bubbles, and then thermally cured at 60 °C in an oven for 3 hrs. After curing, the PDMS stamp could be peeled off from the silicon master. Finally, the PDMS and the glass wafer were bombarded by oxygen plasma at 50 W for 3 min to modify their

functional groups of surfaces from hydrophobic to hydrophilic temporarily. Then the channel of the PDMS was covered and glued on the glass to form a sensor chip.

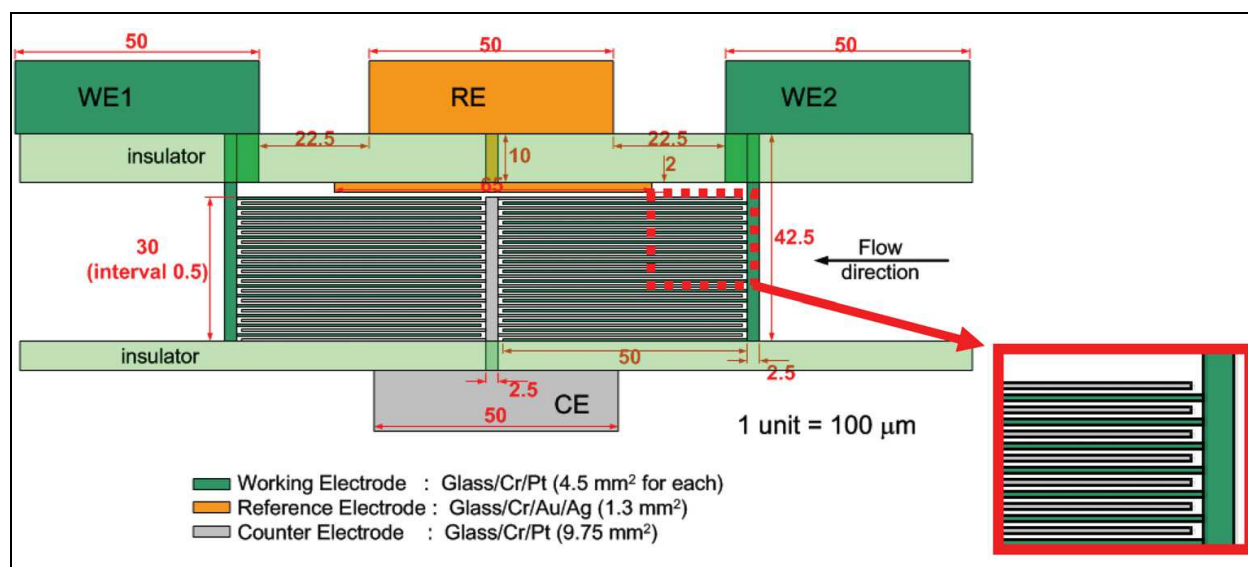


Fig. 3. The schematic of the microelectrode.

For the purpose of getting a more stable reference, the silver surface of reference electrode was modified to Ag/AgCl by chemical deposition. According to Eq. (7), the Ag surface was oxidized to form a thin layer of AgCl in 0.1 M FeCl₃ solution spontaneously for 1 hr. For the enzyme-modified working electrode, the conducting polymer, PEDOT, was prepared in a flow system and the other conditions are the same as described in our previous work (Nien et al., 2006). The PEDOT film was obtained by electropolymerization of EDOT with the sweeping potential from 0.2 to 1.2 V for 20 cycles at a flow rate of 5 ml/hr and it was used as a matrix to entrap the glucose oxidase for immobilization on "WE1" (in Fig. 3) in a 0.02 M PBS electrolyte containing 2,000 U/ml glucose oxidase and 0.3 M KCl. After electropolymerization, the 0.02 M PBS solution was allowed to flow in the channel for some time to wash out the residuals. The sensing chip was stored at 4 °C when not use.



3.3 Fabrication of system B

The substrate, ssteel, was cleaned in the alcohol and water by supersonic wave to remove the organic matter. Then the carbon paste was roll coated on the substrate with constant spacer and area of 1 x 1 cm², which is a large area comparing to literatures, and thermally cured in the oven at 130 °C for 2 hr to remove solvent. Afterward, the mediator, BZQ (50 mM) dissolved in dimethylformamide (DMF), and the GOD dissolved in water (5000 U/ml) were both dropped a volume of 40 μl on the ssteel/C electrode to dry in order. At last, the conducting polymer, PEDOT was electropolymerized on the outer layer of the prepared ssteel/C/BZQ-GOD electrode to prevent the leakage of GOD, in a pH 7 phosphate buffer solution containing 10 mM EDOT monomer and 0.1 mM non-ionic surfactant, PEG, by applying a constant potential of 1.2 V vs. Ag/AgCl/sat'd KCl for 50 s. Afterward, the ssteel/C/BZQ-GOD/PEDOT electrode was stored in a pH 7 PBS at 4 °C when not in use.

4. Results and discussions of system A

4.1 The sensing chip

In each 4-in glass wafer, two pieces of sensing chips (70 mm × 35 mm) were cut by a diamond cutter. The microelectrode arrays can be divided into four sections (mentioned in section 3.2) and the surface metal layer of the working, reference and counter electrodes are platinum, silver and platinum, respectively. The SEM pictures (not shown) indicated that the dimension of the interdigitated array was the same as that of the designed pattern. Finally, by covering the PDMS with a microfluidic channel of 100 μm height, the sensing chip was fabricated. Moreover, for getting a better stability of long-term operation, the surface of reference electrode was modified as Ag/AgCl by chemical deposition. The open circuit voltage (V_{oc}) of the Ag/AgCl is about 95 mV vs. SCE (commercial model) in the electrolyte of 0.3 M KCl and the variation between the two electrodes is less than 5 mV for a period of 2,500 s. It was approximately corresponding to the theoretical value of 102.9 mV based on the electrochemical theory.

When the potential of the first working electrode (WE1) was cycled between a potential range of 0.2 ~ 1.2 V at a scan rate of 0.1 V/s, the current for the first cycle increased at around 0.7 V due to the oxidation of the EDOT monomer. On electrooxidation, a radical cation of EDOT is produced which is transformed to a polymeric species via several follow-up reactions. However, the anodic current at higher potential (0.9-1.2 V) decreased with the cycle number, because the high potential may result in the partial degradation (Fabiano et al., 2002) or overoxidation of PEDOT film. In the 15th to 20th cycles, the CVs of polymerization were almost the same and this implies that the polymer film was not growing due to the resistance of polymer film. During the polymerization process, the PEDOT possesses positive charges, so the negatively charged glucose oxidase (pI=4.2) would migrate to the PEDOT surface at pH=7.4 (PBS) and be grabbed by the growing polymer chains. Besides, it is also an advantage of CV method that the enzyme has more time to diffuse to the polymer surface when the applied potential was swept to the cathodic direction in which the EDOT can't be polymerized. The immobilized enzyme was quantified as about 0.101 U/cm² by UV spectrophotometer after series chemical reactions in previous work (Nien et al., 2008).

4.2 The sensing performance

In Fig. 4, the flow injection data were obtained by applying 0.7 V at a flow rate of 10 ml/hr and each current pulse was resulted from different concentrations with an injecting volume of 30 μl. Besides, both the peak current and the charge capacity of each pulse can be collected as the sensing signal. However, the reproducibility in the peak currents was not good and hence the charge capacities were used for recording the sensing signal instead of current. The relationship obtained between the net charge capacities and the different glucose concentrations by applying a voltage of 0.7 V vs. Ag/AgCl on PEDOT modified enzyme electrode (WE1) with the same injecting volume of 30 μl at different flow rates is shown in Fig. 5. The linear regression falls from 1 to 10 mM, which includes the range of normal human blood, with a sensitivity of 157 μC cm⁻² mM⁻¹ (4.6 μA cm⁻² mM⁻¹ in current plot, which is not shown). The sampling time, the time taken from each injection of the sample to the pulse current returning approximately to the background level, was within 180, 100, 70 and 30 s at a flow rate of 5, 10, 20 and 50 ml/hr, respectively. Operating at a high flow rate had a faster response, but lost another important parameter, sensitivity. Hence the

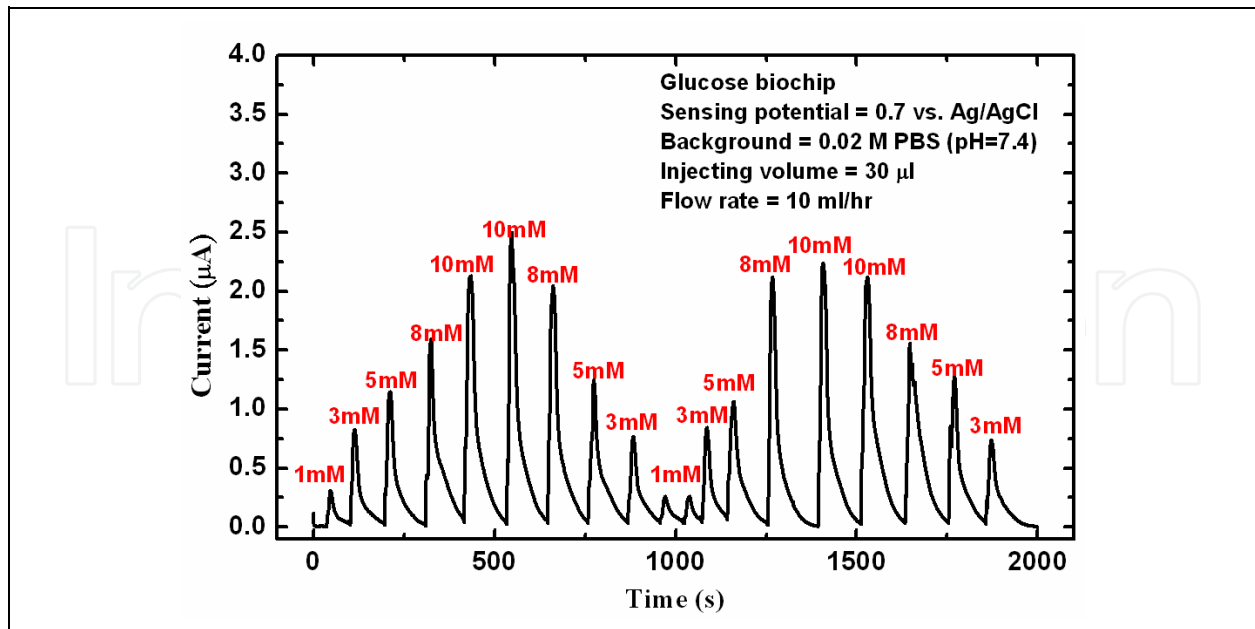


Fig. 4. The sensing signals of the biosensor in response to various glucose concentrations using flow injection analysis.

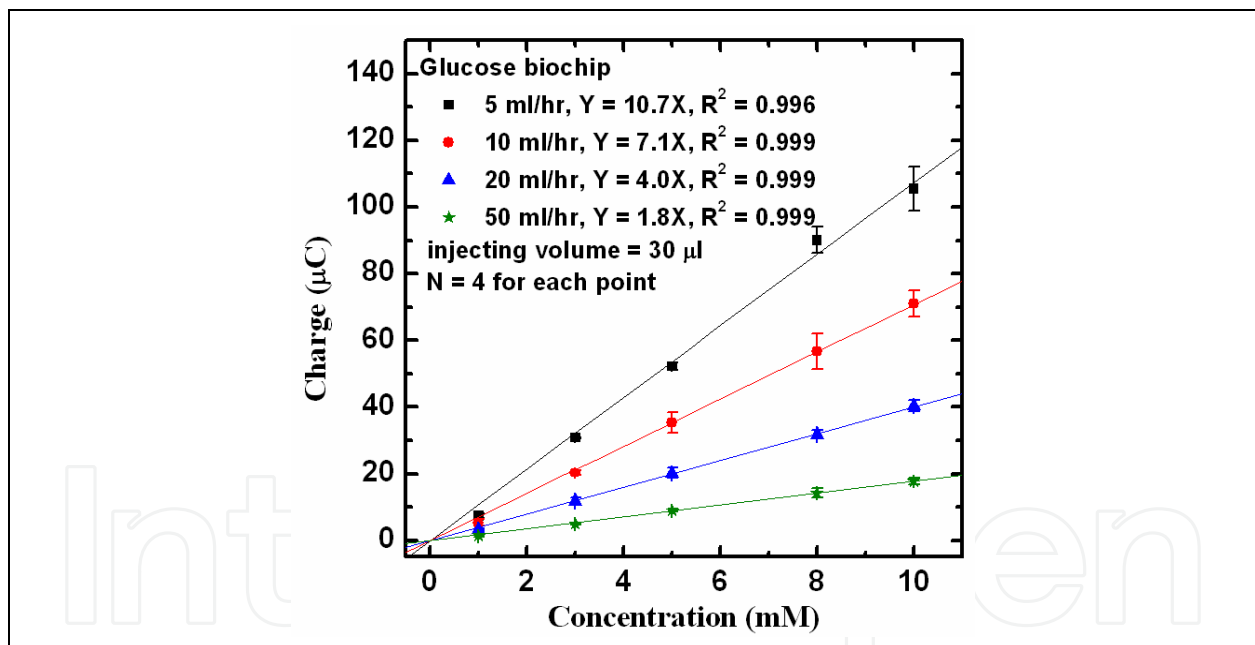


Fig. 5. The calibration curve based on charge at different flow rates.

proper flow rate was selected as 10 ml/hr by considering the performance. The response time and the recovery time, defined as the time taken for the current reaching of 95% of the steady-state level, are about 15 s and 35-75 s, respectively. For different concentrations of the samples, the response time is almost the same, but the recovery time varies with the concentration. The higher concentration of sample leads to longer recovery time, since the larger driving force of mass transport results in a broader concentration profile. Additionally, the limit of detection (LOD) based on signal to noise ratio equaling to 3 is 0.15 mM at 10 ml/hr.

Table 2 is a partial list of the amperometric GOD-based glucose sensors, obtained from the literatures based on PDMS chips. They used capillary electrophoresis to separate the interferences (Liu et al., 2006; Zhang et al., 2006a) and immobilized lactate oxidase to catalyze the ascorbic acid on the upstream (Kurita et al., 2002). Besides, the sensing chip (Huang et al., 2007) can not only detect glucose concentrations but also inject insulin automatically. In the aspect of sensing performance, the linear ranges almost covered the normal human range (3.5-8 mM), but system A provided fast response and recovery times. However, the limit of the detection was not as low as the others, and hence the applications may have some limitations.

References	Immobilized method	Sensitivity ($\mu\text{A cm}^{-2} \text{ mM}^{-1}$)	Linear range (mM)	Response time (s)	Recovery time (s)	LOD (μM)
(Zhang et al., 2006a)	adsorption	---	0-30	10	15	6.5
(Liu et al., 2006)	adsorption	0.0312	0.01-5	---	---	5
(Yamaguchi et al., 2002)	entrapment	8.67	0-20	50	---	-
(Kurita et al., 2002)	entrapment	0.0025	0.01-1	120	---	2.3
(Huang et al., 2007)	entrapment	0.0076	2-30	50	---	---
System A	entrapment	4.6	1-10	15	35-75	150

Table 2. Partial literatures of the amperometric GOD-based glucose sensors on PDMS chips.

4.3 The interferences effect and the monitoring of real sample

One of the most important problems to tackle for any practical application of amperometric biosensors is to minimize the effect of interfering substances possibly present in a real sample. For oxidase-based systems, reductants are the most severe interferences and among these ascorbate and uric acid (Navera et al., 1993; Vasantha & Chen, 2006) are two of the most considered. Moreover, well-separated voltammetric peaks were observed for dopamine and ascorbate anion at the PEDOT modified electrodes at 0.21 and 0.08 V, respectively (Matuszewski et al., 1990). Although the linear range covers the glucose level of normal human, the oxidation-favored interferences in blood, such as ascorbic acid (AA) and uric acid (UA), still raise the sensing currents at a high voltage of 0.7 V. To solve this, the pre-reaction section, "WE2" in Fig. 3, was designed to reduce the interference effect in this microsystem. Here, the bi-potential was applied simultaneously to both WE1 and WE2 by the bi-potentiostat of CHI 900. In the flow injecting analysis, when the samples were injected, the oxidation-favored substances near the boundary layer were oxidized at 0.7 V (vs. Ag/AgCl) on WE2. Consequently, the interferences near the boundary layer were partially eliminated to some extent before the analyte arriving to WE1. The electrode gap between WE1 and WE2 (0.25 mm) is designed to be very close so as to avoid the solution diffusing from the outside boundary layer into the inside layer. For a single-potential test on WE1, both 0.08 mM AA sample and a blend of 0.08 mM AA plus 10 mM glucose sample reached 31.3% and 145.5% of the sensing current obtained for 10 mM glucose, respectively. AA contributes a significant current to the total current on the PEDOT enzyme-modified electrode. In contrast, the sensing current of the blend sample reached 99.6% that of 10 mM glucose response in a bi-potential test. The result shows that the concentration of the oxidation-favored species in the sample was reduced appreciably. Therefore, it is concluded that the bi-potential configuration can reduce the interferences in a flow injection system

and thereby improving the selectivity and specificity of an enzyme modified electrode towards glucose oxidation.

For the real sample test, three different methods were used to monitor the glucose concentration of the human blood. One was determined by a hospital, another was obtained by a handheld commercial product (EasiCheck blood glucose test strips) and the third was detected by this sensing chip. After the oxidation of the interferences on WE2, the reduced concentration of the interferences in the boundary layer can enhance the accuracy of glucose sensing for WE1. The results obtained from the three methods are shown in Table 3 and the percentages of the detecting error were calculated according to the value obtained from hospital as a standard. Moreover, the bias of the bi-potential (+13.6%) was much lesser than that of single-potential (+141%), and this confirms that the WE2 helps to eliminate the interference. For the commercial product, the average concentration of three tests was 4.77 mM and the bias was about +5% which is within the bias range ($\pm 20\%$) of the product prescription. Although the error of the bi-potential was acceptable grudgingly, it may be lowered further by increasing the active area of WE2.

Glucose biochip	Commercial product	NTU Hospital
Single-potential = 10.99 mM (Bias = +141%)	4.77 mM (Bias = +4.6%)	4.56 mM (as a standard)
Bi-potential = 5.18 mM (Bias = +13.6%)		

Table 3. A summary of the detecting errors for a real sample tested against different methods.

5. Results and discussion of system B

5.1 The enzyme electrode

For the stable test of BZQ adsorbed inside the electrodes, it was under the sweeping potentials between -0.6 and 0.8 V for 100 cycles. With increasing cycle numbers, the peak currents of redox reaction were decreased to stable values after about 50 cycles (not shown). This implied that the electrodes can reach to a stable situation after the leakage of the weakly-adhesive BZQ. It showed that the first layer, carbon, was a good substrate for the adsorption of BZQ. For the third layer, the GOD dry-coated on the electrode may dissolve into the electrolyte while the step of electropolymerization. As a result, the immobilized amount of GOD can be quantified by the absorbance change of the electrolyte before and after polymerization. According to the UV spectrum (not shown) of the electrolyte at 280 nm, which is the maximum absorbent wavenumber for GOD, the leakage of GOD in the electrolyte was calculated to be about 132 U and the entrapped efficiency was about 33% for the electrode B. The immobilized amount of GOD by this method is about 68 U/cm² which is higher than 0.101 U/cm² of the GC electrode by entrapping enzyme and polymerizing PEDOT at the same time reported in the previous work (Nien et al., 2006).

5.2 The sensing performance

For glucose biosensors, the prepared electrode worked in the nitrogen purged glucose solutions at a stirring rate of 100 rpm by applying a sensing potential of 0.3 V to record the oxidation sensing current of BZQ. The calibration curve of the electrode was shown in Fig. 6

and the sensitivity was $2.21 \text{ mA M}^{-1} \text{ cm}^{-2}$ with a R^2 value of 0.993. Besides, the other performances including linear range, response time and limit of detection were 1.1-15 mM (human range included), 95-105 s and 1.1 mM, respectively. Comparing to the previous works of co-immobilization of GOD and mediators, the sensitivity is better than 0.79 (Himuro et al., 2009), 0.111 (Crespilho et al., 2008) and 1.86 (Qiu et al., 2007) $\text{mA M}^{-1} \text{ cm}^{-2}$. In the first work, the GOD was linked on the mediator-based copolymer backbone, poly(vinylferrocene-co-2-hydroxyethyl methacrylate), and polyamidoamine particle linked by GOD was co-immobilized with the gold nanoparticles modified by cobalt hexacyanoferrate (mediator) on conducting glass in the second work. In the last work, the GOD and ferrocene monocarboxylic acid-modified Fe_3O_4 nanoparticles were both entrapped on carbon paste electrode. In addition, only the linear range of the first literature (1.4-8.9 mM) covered the normal human range.

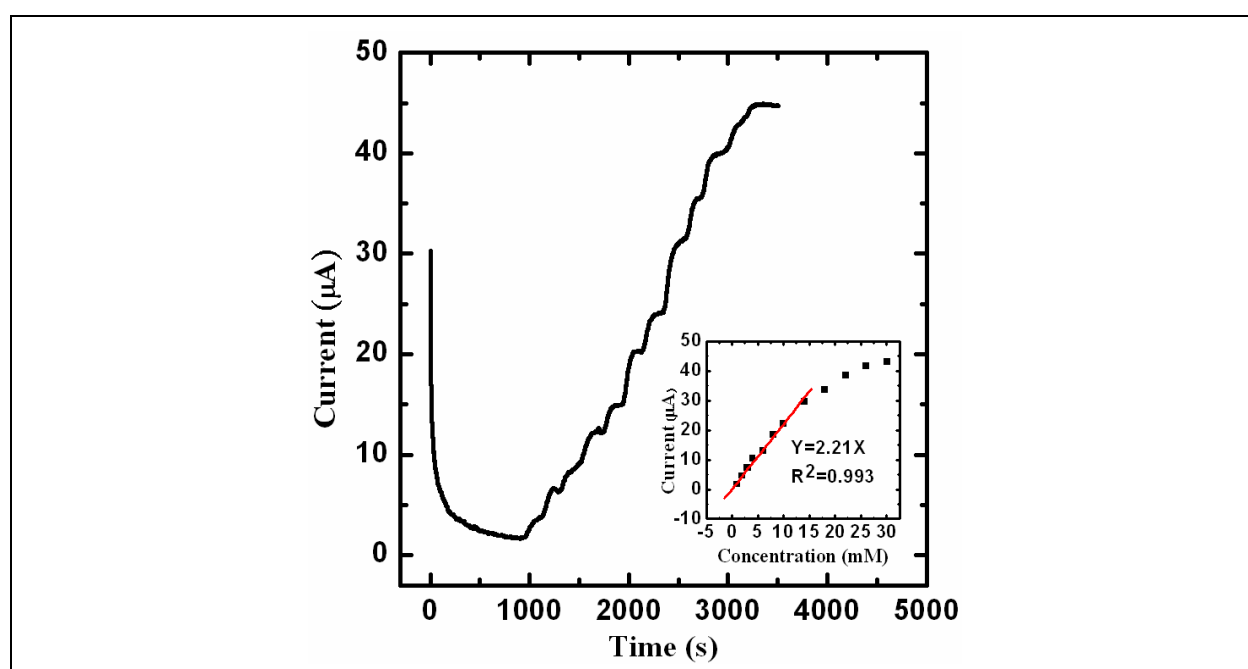


Fig. 6. The current response with increasing glucose concentrations and the calibration curve (inset) for system B.

5.3 The interferences effect and monitoring of real sample

For simulating the real sample, the most common interferences including AA, DA, UA and AP were applied in this system, and their formal potentials are about 0.2 V, 0.3 V, 0.5V and 0.3 V vs. Ag/AgCl/sat'd KCl, respectively. Besides, the normal ranges of those four species in blood are 34-80 μM , $<1 \mu\text{M}$, 178-416 μM and 130-150 μM , respectively. The current responses of the interferences were shown in Fig. 7 based on the sensing current of 6 mM glucose as 100%. In Fig. 7, there is almost no current response for UA owing to the insufficient overpotential. However, the sensing current of DA was higher than that of AA which has a lower oxidized potential. It is because some carbons on the polymer backbone was over-oxidized and transformed into carboxylic groups at a high potential when polymerization (Cosnier, 2003; Vidal et al., 2001). In the literature (Palmisano et al., 1995), the carboxylic groups on the over-oxidized conducting polymer, polypyrrole, were proved by X-ray photoelectron spectroscopy (XPS). In other words, the charge of PEDOT polymer

chain changed to partial negative from positive, so the PEDOT film preferred to attract positive DA ($pK=8.87$) than negative AA ($pK=4.1$) in the PBS of pH 7. Additionally, the AP with positive charge resulted in 3% current response owing to the same way stated above. Finally, the whole blood from human beings was also monitored in a batch system and it shows an bias of +3.6% according to the standard glucose concentration of 4.94 mM obtained from National Taiwan University Hospital. As a result, the modified electrode presents a good performance for real samples detections in an oxygen-independent system.

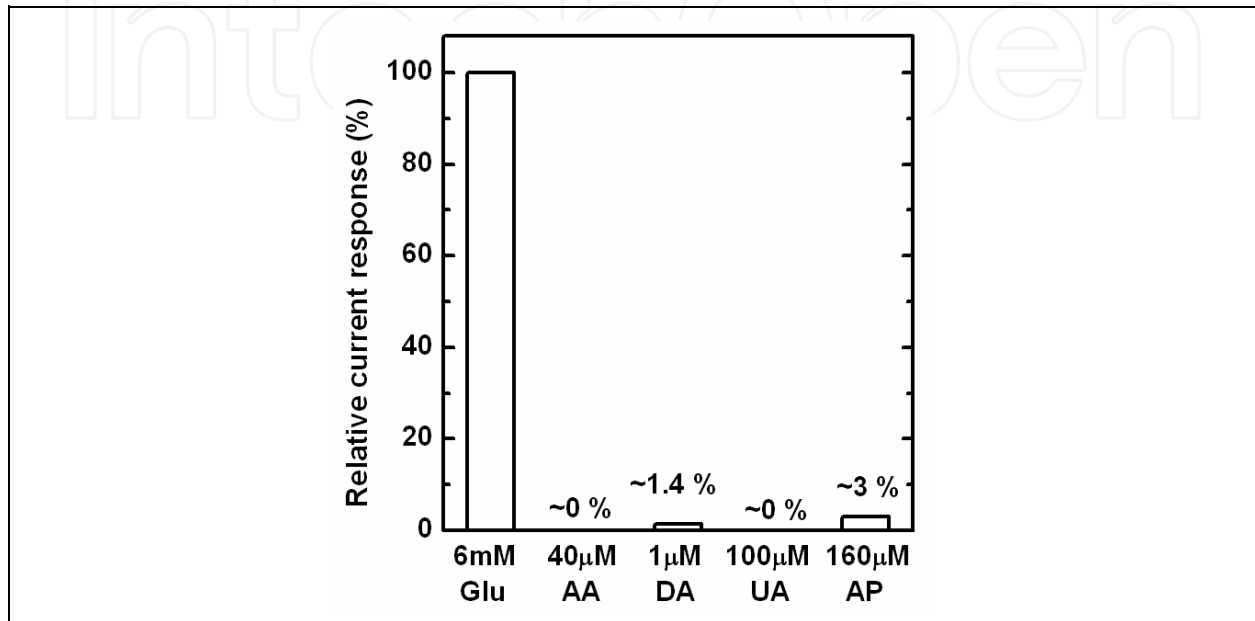


Fig. 7. The relative current response of the common interferences in human blood.

5.4 The application of biofuel cell

The modified electrode was employed for not only as a glucose biosensor but also as an anode for biofuel cell. In the following biofuel cell system proposed, the anode was the modified electrode immobilized BZQ and GOD, and the cathode was the platinum electrode in the ABTS and Lac solution. Figure 8 shows the I-V curves obtained by sweeping potentials from open circuit voltage (V_{oc}) to 0 V and power curves in 0 M and 0.1 M glucose solution at room temperature and body temperature, which means 25 °C and 37 °C, respectively. In the Fig. 8A, the V_{oc} increased to 0.6 V from 0.52 V and the cell current had an obvious enhancement after adding 0.1 M glucose. It implied that the glucose biofuel cell was workable and sensitive to glucose concentration. Based on Fig. 8A, the power curves shown in Fig. 8B can be calculated from the current multiplied by cell voltage. In Fig. 8B, the cell power of the 0 M glucose solution was 7 $\mu\text{W}/\text{cm}^2$ and the cell acted as a non-regenerated cell by the redox reactions of the mediators in each compartments. Besides, the maximum power of 22.5 $\mu\text{W}/\text{cm}^2$ (at $V_{cell}=0.235$ V and $I=95.8$ μA) at body temperature was slightly higher than that of 18.9 $\mu\text{W}/\text{cm}^2$ (at $V_{cell}=0.212$ V and $I=89.1$ μA) at room temperature. It is because the power at 37 °C is affected by the higher catalytic activity of enzyme and the lower fuel solubility of oxygen in cathode.

In this system, the anode was assigned as the rate-determining electrode to optimize the amount of BZQ and GOD. Thus, the catholyte always contains sufficient ABTS and Lac comparing to anodic electrolyte and the maximum reaction rate of cathode was much higher

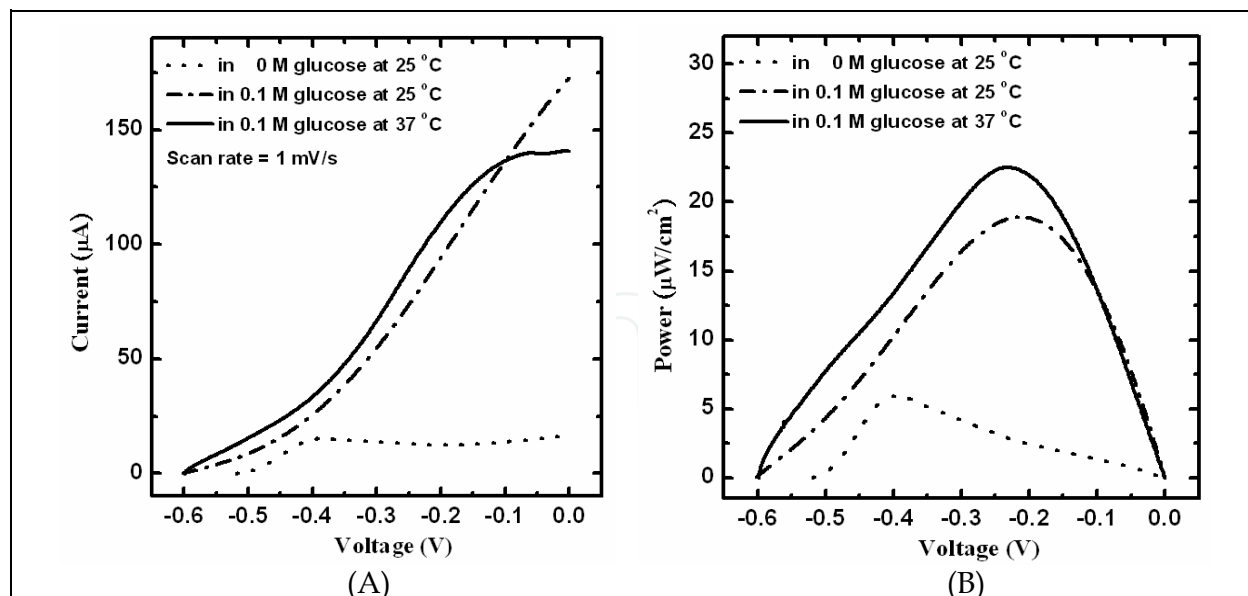


Fig. 8. (A) The I-V curve and (B) the power curve of the biofuel cell at 25 °C and 37 °C.

than that of anode. Figure 9 shows the maximum powers of the cell with the anodes prepared by different amounts of BZQ and GOD at 25 °C. The maximum powers were only varied with the amount of BZQ but GOD in Fig. 9. However, the immobilized content of BZQ was much high according to its redox peak current, and the mole ratio of that to the quantitative GOD (mole of BZQ/mole of GOD) was much larger than 10. It may be due to the poor contact between the BZQ with hydrophobic property and GOD with hydrophilic property. In other words, the electron-transfer reaction may be only carried out in the near-interface of BZQ and GOD layers, so it also resulted in lower sensing current and cell power. Besides, the powers of the electrode prepared by 80 μl BZQ were almost the same as that by 40 μl BZQ, so the optimal condition was 40 μl BZQ and GOD.

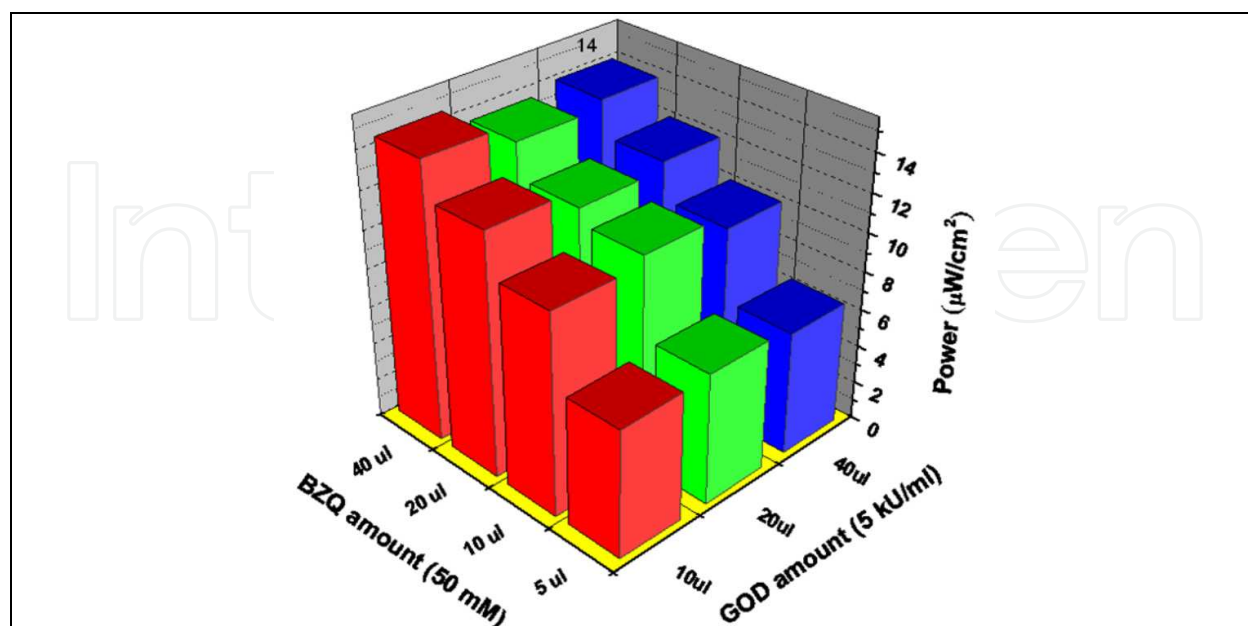


Fig. 9. The maximum cell powers using different anodes prepared by various amounts of BZQ and GOD at 25 °C

Table 4 shows a partial list of the literatures for the biofuel cells immobilized GOD and Lac by physical immobilized methods, such as adsorption and entrapment. In the first three references (Nos. 1-3), the cells with the mediators dissolved in electrolytes were carried out in membrane systems. However, the mediators were immobilized on the electrodes for the last two references (Nos. 4-5). According to Table 4, this system can provide a largest V_{oc} and the maximum power is better than that of some references (Nos. 1, 3, 4).

No.	Reference	Immobilized Method	V_{oc} (V)	Power ($\mu\text{W}/\text{cm}^2$)	
				23 °C	37 °C
1	(Yan et al., 2007)	Lipid based	0.45	3.2	---
2	(Liu & Dong, 2007a)	Gel	0.4	29	---
3	(Liu & Dong, 2007b)	Gel	0.25	10	---
4	(Habrioux et al., 2007)	Adsorption	0.3	---	16
5	(Brunel et al., 2007)	Adsorption	0.3	---	29
6	System B (Nien et al., 2009)	Entrapment	0.6	18.9	22.5

Table 4. A partial list of literatures on the power output of biofuel cells with glucose oxidase and laccase which were immobilized by adsorption and entrapment.

6. Conclusions and future works

In system A, the PEDOT-modified electrode was used as a matrix to entrap glucose oxidase and was integrated in a flow system of sensing chip successfully. The optimal injecting volume and flow rate were 30 μl and 10 ml/hr, respectively. The performances of sensitivity, linear range, response time, recovery time and limit of detection were 157 $\mu\text{C cm}^{-2} \text{mM}^{-1}$, 1-10 mM, 15 s, 35-75 s and 0.15 mM at a flow rate of 10 ml/hr, respectively. With an applied potential of 0.7 V on WE2, it can reduce the interference current of WE1. Since the interferences in the flow channel near the surface of the first electrode (WE2) had been pre-reacted electrochemically, and the interference-free sensor can be achieved at the second electrode (WE1). In the real sample test, the bias of bi-potential was +13.6%, which is lower than that of single-potential. In system B, the proposed electrode fabricated by multilayer structures successfully works as a glucose biosensor in the oxygen-independence solution, and the anode of the biofuel cell by adding not only glucose solution but also the real blood of human beings. The electrode prepared by BZQ/DMF, shows a sensitivity of 2.21 $\text{mA M}^{-1} \text{cm}^{-2}$, a linear concentration range of 1.1~15 mM (including the human blood range) and a response time of 100 s at a sensing potential of 0.3 V. Besides, the current responses of the common interferences in blood were much lower than that of 6 mM glucose because of the low sensing potential and the partially negative charged polymer film. As the glucose/ O_2 biofuel cell, the V_{oc} can reach to 0.6 V and the maximum power was 22.5 $\mu\text{W}/\text{cm}^2$ at 37 °C in 0.1 M glucose solution. For the real blood tests, the bias was about +3.6% comparing to the standard value from hospital in glucose sensing and the cell power was 25 $\mu\text{W}/\text{cm}^2$ in biofuel cell at 25 °C.

Nowadays, most of diabetes check their glucose level by the commercial glucose test strips at home and inject insulin if they need. However, hemoglobin A1c, Hb_{A1c} recommended by the American Diabetes Association (ADA) provides an average blood glucose level during 60-90 days. It is a more accurate biomarker for long-term monitoring without external factors. In literatures, fructosyl valine (FV), which exists after protein digestion of Hb_{A1c} , was

monitored by enzyme-based electrochemical biosensor (Fang et al., 2009) or molecular-imprinting biosensor (Chuang et al., 2009). The recent challenge in Hb_{A1C} part is still how to lower the interference signal, especially the effect of heme in our experiment. Heme also exists along with FV in sample after protein digestion, so this matter will be an issue for future study.

7. Acknowledgement

This work was partially sponsored by a grant received from the Ministry of Economics, Taiwan. Some instruments used in this study were supported by the National Research Council of Taiwan.

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Intelligent and Biosensors

Edited by Vernon S. Somerset

ISBN 978-953-7619-58-9

Hard cover, 386 pages

Publisher InTech

Published online 01, January, 2010

Published in print edition January, 2010

The use of intelligent sensors have revolutionized the way in which we gather data from the world around us, how we extract useful information from that data, and the manner in which we use the newly obtained information for various operations and decision making. This book is an attempt to highlight the current research in the field of Intelligent and Biosensors, thereby describing state-of-the-art techniques in the field and emerging new technologies, also showcasing some examples and applications.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Po-Chin Nien, Po-Yen Chen and Kuo-Chuan Ho (2010). Amperometric Enzyme-based Biosensors for Lowering the Interferences, Intelligent and Biosensors, Vernon S. Somerset (Ed.), ISBN: 978-953-7619-58-9, InTech, Available from: <http://www.intechopen.com/books/intelligent-and-biosensors/amperometric-enzyme-based-biosensors-for-lowering-the-interferences>

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