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A Mediated Glucose Biosensor Incorporated with Reverse Iontophoresis Function for Noninvasive Glucose Monitoring

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Abstract—The objective of this study was to develop a ferrocene mediated glucose biosensor for reverse iontophoresis. An amperometric ferrocene mediated glucose biosensor based on a three electrodes planar configuration was constructed using screen printing technique. Different combinations of glucose oxidase and ferrocene loading were drop coated onto the surface of the amperometric transducer. The amperometric transducer was characterized electrochemically using cyclic voltammetry and its electrochemical characteristics ($\Delta E_p = 70$ mV, $I_{pa}/I_{pc} = 0.89$) were found close to an ideal amperometric transducer. The biosensor on the detection of glucose at 200 mV (vs. Ag/AgCl) showed a linear response range (0–4 mM). The response time of the biosensor was about 10 s. Finally, the biosensor was used together with reverse iontophoresis technique. By the use of an actual model for evaluation, an excellent linear relationship ($r^2 = 0.99$) was found between the glucose concentration of the actual model and the biosensor current response. In conclusion, a ferrocene mediated glucose biosensor incorporated with reverse iontophoresis function was developed.

Keywords—Amperometric, Ferrocene, Glucose, Biosensors, Reverse Iontophoresis.

INTRODUCTION

Amperometric biosensor based on oxidase enzymes that generate peroxide (H_2O_2) are the most widely used biosensors, the transduction path being the electrochemical oxidation of the peroxide formed in an enzyme reaction.[7] The development of glucose amperometric biosensors is related to a primary commercial purpose and many efforts have been devoted to the fabrication of compact portable units for personal use by diabetics.[57]

However, the generated peroxide is harmful for glucose oxidase (GOD) and limits the performance of the biosensor. One viable solution is to replace the natural electron acceptor (dioxygen, O_2) of glucose oxidase by electroactive compounds that will act as redox mediators.[6] An enzyme electrode based on an electron transfer mediator was reported by Cass *et al.* 20 years ago.[6] To date the most popular and possibly the most reliable types of biosensors reported on in the literature and utilized commercially are those employing redox enzymes coupled with amperometric detection.[12,36] Biosensors based on mediators have been reported, such as ferrocene derivates,[3,6,55] tetrathiafulvalene[1,25] and viologen derivatives.[27] Among the mediators investigated, ferrocene and its derivatives are the most successful mediators due to their various very desirable properties, e.g., relatively low molecular mass, good stability in solution, reversibility, regeneration at low potential, independent pH, unreactivity with oxygen, and generation of stable redox forms.[18,46,47] Therefore, ferrocene was used as a mediator in this work.

Skin provides a unique gateway for noninvasive transdermal monitoring. Reverse iontophoresis refers to the passage of a low level of current through the skin to promote the extraction of both charged and neutral molecules. Recently, reverse iontophoresis techniques have been used for patient monitoring.[14,43–45,50,53] To the best of our knowledge, only one glucose sensor was reported in the literatures incorporated with reverse iontophoresis function.[4,15–17,19,22,23,28,32,33,40,43,48,50–54] This glucose biosensor is the Food and Drug Administration (FDA) approved GlucoWatch® biographer which proposes a solution for diabetics to avoid the

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frequent need for blood sampling. The GlucoWatch® biographer, originally produced by Cygnus, Inc. in Redwood City, CA, who sold the rights to Animas, is the first real-time continuous glucose monitor introduced. The GlucoWatch® passes a small current between two skin-surface hydrogel electrodes in order to extract glucose-containing interstitial fluid into hydrogel pads incorporating a glucose oxidase biosensor.[23,49,50] However, the GlucoWatch® causes skin rash with irritation under the device in many patients. This may be due to the immobilization of glucose oxidase inside the hydrogel pads and therefore the generated peroxide can easily contact and irritate the user's skin. Moreover, the GlucoWatch® is unable to reliably detect hypoglycemia and hyperglycemia.[15] On the other hand, numerous studies of ferrocene mediated glucose biosensors have been reported on in the literature,[6,11,20,24,35,39,42,58] but none were found on studying ferrocene mediated glucose biosensor incorporated with reverse iontophoresis function. Therefore, the aim of this study was to develop a ferrocene mediated glucose biosensor for reverse iontophoresis.

In this paper, the optimum combination of glucose oxidase and ferrocene was found in order to develop a mediated glucose biosensor incorporated with reverse iontophoresis function for noninvasive glucose monitoring. The glucose biosensor for reverse iontophoresis was finally evaluated in this study.

MATERIALS AND METHODS

Reagents and Solutions

All reagents were commercially available and were employed without further purification. Ferrocene, toluene, 1-cyclo-hexyl-3-(2-morpholinoethyl)carbodiimide-*p*-methyltoluenesulphate, glucose oxidase (GOD), D(+)-glucose, phosphate buffered saline (PBS) tablet, ferrocenemonocarboxylic acid (FMCA), sodium perchlorate, acetate buffer were purchased from Sigma Chemical Co. (St. Louis, MO). Methylcellulose (MC), Methocel A4M Prem, was purchased from Dow Chemical Co. (Midland, MI). De-ionized water that had been purified by a Millipore System (Milli-Q UFplus; Bedford, MA) was used to prepare all solutions.

Graphite paste, silver paste, and silver-silver chloride (Ag/AgCl) paste were purchased from Advanced Conductive Materials (Atascadero, CA). Insulating paste (Polyurethane) was purchased from Measurement Group UK Ltd (Hants, UK). Polyethylene terephthalate (PETE) sheet was purchased from 3 M.

Construction of the Amperometric Transducer and Ferrocene Mediated Glucose Biosensor

The construction steps of a three-electrode amperometric transducer were shown schematically in Fig. 1. The transducers were constructed using the conventional screen printing technique (screen mesh size = 390 counts per inch; screen emulsion thickness = 25 μm). There were four different layers for each transducer: Conducting tracks, insulation shroud, Ag/AgCl pads and graphite pads. These four different layers were printed on a clear PETE sheet one after the other and each layer were allowed to dry at room temperature for 2 h. Prior to the screen printing process, the PETE sheets were kept at 80 °C for 1 h annealing. Silver paste was firstly used to print the conducting tracks. Parts of the conducting tracks were

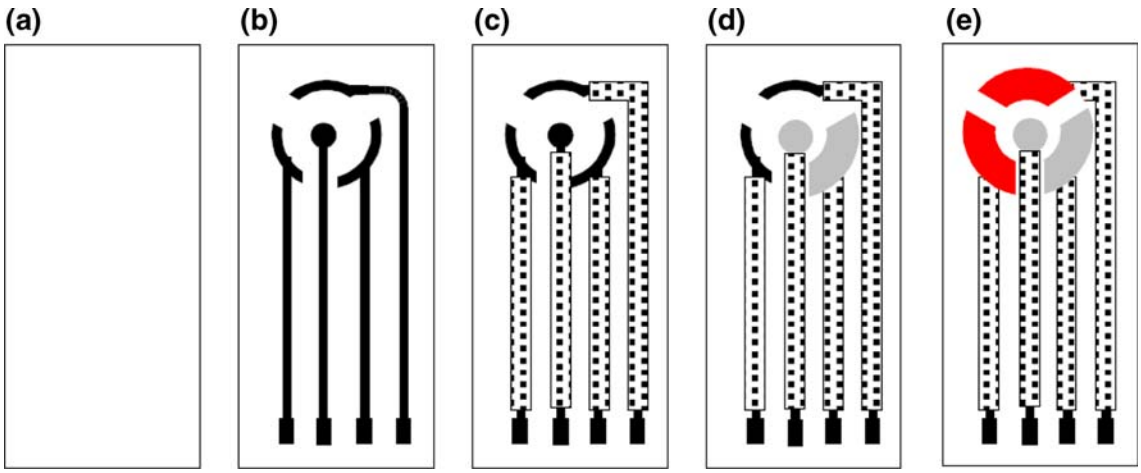

FIGURE 1. Construction steps for the planar configuration of the screen-printed amperometric transducer. (a) PETE support material; (b) printing of conducting silver basal track; (c) printing of insulation layer; (d) printing of Ag/AgCl pads, the central circular pad is iontophoresis electrode for reverse iontophoresis while the other pad is reference electrode; (e) printing of graphite pads which are working and counter electrodes.

then covered by an insulation shroud of insulating paste. Ag/AgCl paste was subsequently applied to the end of conducting tracks to form Ag/AgCl pads (reference and iontophoresis electrodes). Graphite paste was finally applied to the end of the remaining conducting tracks to form graphite pads (working and counter electrodes).

The same transducer fabrication procedures mentioned above was used for ferrocene mediated glucose biosensor construction. A modified method from Cass[5] was used to construct the glucose biosensor. 0–0.2 M Ferrocene (in toluene), 0.15 M 1-cyclo-hexyl-3-(2-morpholinoethyl)carbodiimide-*p*-methyltoluene-sulphate (in 0.1 M acetate buffer pH 4.5) and 4.5–16.5 mg/mL glucose oxidase (in 0.1 M acetate buffer pH 4.5) were drop coated on the surface of one graphite pad (working electrode) of the transducer successively and dried for 90 min at room temperature. The volume of each solution dropped was 10 μL. A silicon O-ring (10 mm thickness) was then fixed on the surface of the glucose biosensor. The glucose biosensors were kept under 4 °C when not in use.

Cyclic Voltammetry Measurements of the Amperometric Transducer

The amperometric transducer was connected to a potentiostat (electrochemical interface SI1286, Schlumberger Technologies, England) coupled to a Corrware software (Schlumberger Technologies, England). A solution (500 μL) of 0.5 mM FMCA in 0.01 M PBS pH 7.4 with 100 mM sodium perchlorate was added onto the surface of the transducer. The cyclic voltammetry was recorded between −0.1 and +0.5 V using a scan rate of 100 mV/s.

Measurements of Current Response to Glucose

The ferrocene mediated glucose biosensor was connected to the potentiostat and a potential of 200 mV vs. Ag/AgCl pad was applied to the working electrode. A solution (50 μL) of 0.01 M PBS pH 7.4 was added onto the surface (inside the silicon O-ring) of the glucose biosensor. After the current became steady, a 200 μL of different glucose concentration solution was injected inside the silicon O-ring. The working mechanism of ferrocene on glucose biosensor is shown in Fig. 2.

Evaluation of the Ferrocene Mediated Glucose Biosensor for Reverse Iontophoresis

A 90 μL of a 4% MC gel (prepared by mixing 4 g of MC with 100 mL of 0.1 M PBS) was pipetted to two biosensors, which had the optimum combination of glucose oxidase and ferrocene, at the geometrically defined windows formed by the silicon O ring. The two biosensors (23 mm apart between the iontophoresis electrode centers) were then placed onto the nanoporous membrane (Spectra/Por® CE, MWCO: 500, Spectrum Laboratories, Inc., Canada) of our own developed diffusion cell[10] and were fixed in position with adhesive tapes. The diffusion cell was filled with different concentration of glucose solution. By the use of our own developed reverse iontophoresis device,[9] a bipolar current (period = 30 min, i.e., 555.6 μHz) of 0.3 mA/cm^2 was passed between the iontophoresis electrodes at room temperature (22–24 °C) for a period of 90 min. At the end of the experiment, biosensors were connected to the potentiostat for current response measurement and a potential of 200 mV vs. Ag/AgCl pad was applied to the working electrode.

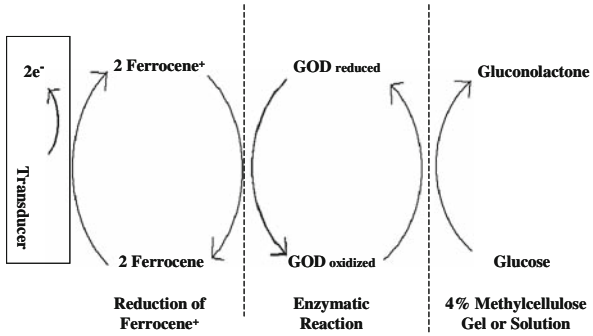

FIGURE 2. The working mechanism of ferrocene on glucose biosensor.

Statistics

One-way analysis of variance (ANOVA) was used to analyze the simple main effect for glucose oxidase loading and ferrocene loading. All statistical analyzes were carried out using SPSS v.11.5 software (SPSS Inc., Chicago, Illinois, USA) with the level of statistical significance set at 0.05.

RESULTS AND DISCUSSION

The Amperometric Transducer

One of the major features of the counter electrode material is to have a good inert behavior. Thus, lots of counter electrode materials, such as gold, platinum,[13,31] etc. have been developed. Galan-Vidal *et al.*[21] reported that there are no dependence between the current density and the materials and geometries used and therefore graphite was used as counter electrode material in this study because of low cost.

The ratio of the counter electrode area to working electrode area (A_C/A_W) has an influence on supplying a determined current to the working electrode without limiting its response.[2] It has been reported that a suitable functioning of the counter electrodes can be achieved by increasing the counter electrode area with respect to the working electrode area. Several works have been reported with the optimal A_C/A_W ratios between 1 and 12.[26,30,34,37,56] Moreover, Galan-Vidal *et al.*[21] reported that the composite material can permit the fabrication of a not activated graphite counter electrode with an A_C/A_W ratio of at least 0.125. Therefore, in this study, the A_C/A_W ratio of 1 was employed on constructing the amperometric transducer because there is no great difference on the current density of transducer constructed with A_C/A_W ratios between 0.11 and 9.20.[21]

The electrochemical characteristic of the amperometric transducer was evaluated from the cyclic voltammetry obtained for the FMCA system. It was found that the transducer had a high response current but a low potential position of the oxidation peak and reduction peak (see Fig. 3 and Table 1). A small separation between both anodic and cathodic peaks means that a fast electron transfer takes place and this resulted in our glucose biosensor having a short response time. As shown in Table 1, the averaged peak potential separation between the anodic and cathodic peak (ΔE_p) and the ratio of the anodic peak current to cathodic peak current (I_{pa}/I_{pc}) were found close to that of an ideal amperometric transducer reported by Nicholson and Shain,[38] i.e., ideal ΔE_p and I_{pa}/I_{pc} should be 59 mV and 1, respectively.

The reproducibility in the construction of the amperometric transducer was evaluated from standard deviation of ΔE_p and I_{pa}/I_{pc} ratio. As shown in Table 1, the relative standard deviation of ΔE_p and I_{pa}/I_{pc} ratio indicated a batch reproducibility of transducer construction of about 6% ($n = 3$).

The Ferrocene Mediated Glucose Biosensor

Figure 4 showed a current–time curve after the addition of glucose solution onto the biosensor. Immediately after the addition of glucose solution, the reductive current increased and reached 95% of the steady state current within 10 s. The key factor for achieving such fast response can be ascribed to the intimate contact between GOD, ferrocene and sensing sites, resulting in rapid establishment of a steady state due to low diffusion barriers. In this study, GOD was bonded to the working electrode by 1-cyclo-hexyl-3-(2-morpholinoethyl)carbodiimide-*p*-methyltoluene-sulphate and this resulted in a very thin glucose sensing layer. Thus the distance between the electrode and the reaction center of the enzyme was very small. Also, owing to the use of ferrocene, our glucose biosensor had a good contact between the redox site and reaction

TABLE 1. The electrochemical characteristics of the amperometric transducers.

Transducer	E_{pa} (V)[a]	E_{pc} (V)[b]	I_{pa} (μA)[c]	I_{pc} (μA)[d]	ΔE_p (V)[e]	I_{pa}/I_{pc}
1	0.281	0.211	6.50	6.97	0.07	0.93
2	0.277	0.213	6.42	7.09	0.06	0.91
3	0.275	0.212	6.82	8.16	0.06	0.84
Mean					0.07	0.89
SD					0.00	0.05
Relative SD					5.77	5.60

[a] E_{pa} is the anodic peak potentials.
[b] E_{pc} is the cathodic peak potentials.
[c] I_{pa} is the anodic peak currents.
[d] I_{pc} is the cathodic peak currents.
[e] ΔE_p is the peak separation ($\Delta E_p = E_{pa} - E_{pc}$).

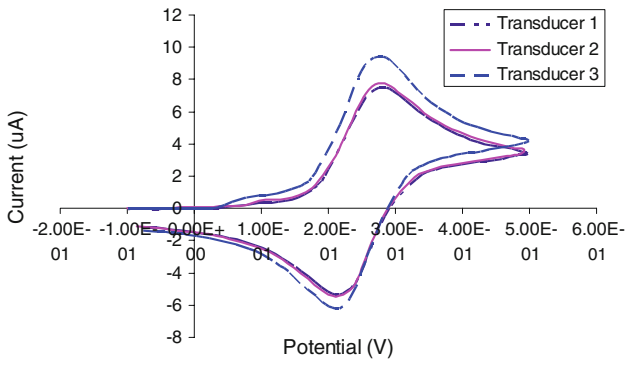

FIGURE 3. Cyclic voltammogram of amperometric transducers. The supporting electrolyte is a 0.5 mM FMCA in 0.01 M PBS pH 7.4 with 100 mM sodium perchlorate. Scan rate is 100 mV/s.

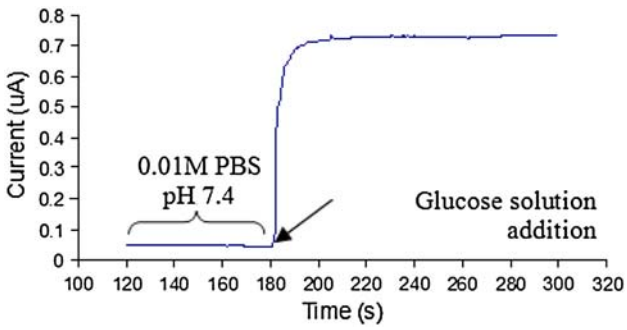

FIGURE 4. Current–time curve obtained by the addition of 4 mM glucose solution on the ferrocene mediated glucose biosensor. Data is obtained from biosensor with the combination of 12.5 mg/mL glucose oxidase and 0.1 M ferrocene.

center of enzyme.[6,41] As a result, the time required to reach 95% of the steady state current was relatively short. The response time of our mediated glucose biosensor is comparable to that reported by Wu *et al.*[58] and is faster response as compared with various mediated glucose biosensors (15–30 s).[11,21,24,35,42]

The magnitude of current response was plotted against the glucose concentration (see Fig. 5) and the sensitivity of the biosensor was determined by the slope of linear regression of this current response vs. glucose concentration plot. It was found that the response current increased with increasing concentration of glucose and the reductive current response was proportional to the glucose concentration from 0 to 4 mM. Since the amount of transdermal glucose extraction by reverse iontophoresis is in the order of μM,[52] the range of measurement of our glucose biosensor can cover the concentrations of the transdermally extracted glucose.

The mean values of the sensitivity for each combination of glucose oxidase loading and ferrocene loading were given in Table 2. The changes in the sensitivity with different concentration of glucose oxidase and ferrocene were also illustrated (see Fig. 6). As shown in Fig. 6, it was found that the sensitivity increased with increasing concentration of ferrocene. Moreover, the sensitivity of the ferrocene mediated glucose biosensor was found to be significantly higher than unmediated glucose biosensor ($p < 0.05$). Unmediated glucose biosensor had a lower sensitivity because it needs dioxygen for its reaction to be taken place. However, ferrocene mediated glucose biosensor does not need dioxygen for its reaction because ferrocene replaces dioxygen as a cofactor for glucose oxidase and thus acts as an electron acceptor.[6] Also, ferrocene enhances our glucose biosensor having a good contact between the redox site and reaction center of enzyme.[6,41] Therefore, this resulted in ferrocene mediated glucose biosensor having a higher sensitivity.

As shown in Fig. 6, there was no great influence on the sensitivity with increasing glucose oxidase loading except the biosensor with 0.1 M ferrocene. For biosensor with 0.1 M ferrocene, the sensitivity of

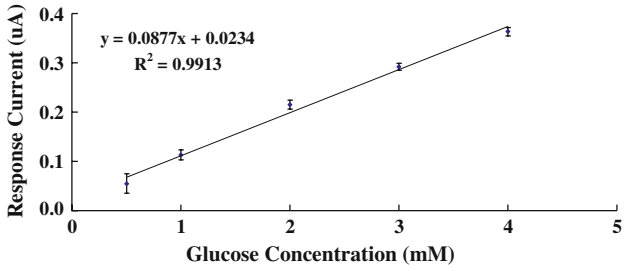

FIGURE 5. Calibration curve of glucose obtained on the ferrocene mediated glucose biosensor. The line is that of best fit found by linear regression ($r^2 = 0.99$). The sensitivity of the biosensor (sensitivity = 87.7 nA/mM) is the slope of the linear regression line. Data is obtained from biosensors ($n = 3$) with the combination of 8.5 mg/mL glucose oxidase and 0.05 M ferrocene.

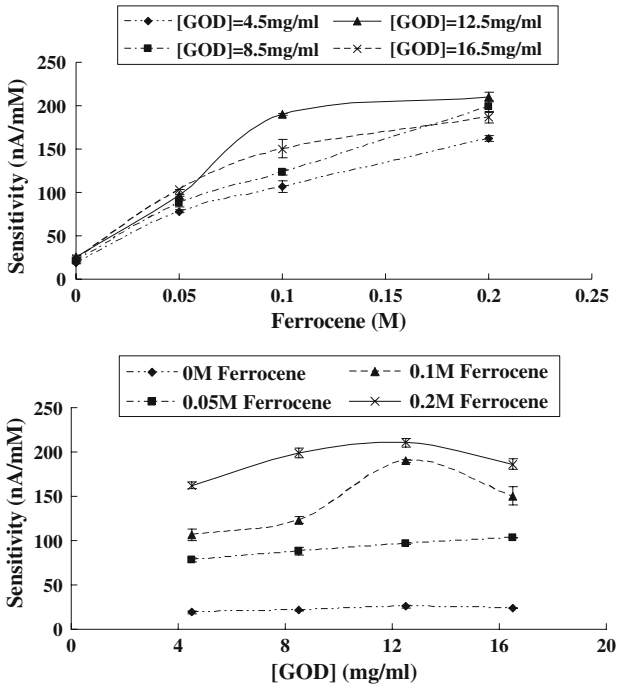

FIGURE 6. The changes in the sensitivity of the ferrocene mediated glucose biosensor at different combinations of glucose oxidase loading and ferrocene loading ($n = 3$).

TABLE 2. The sensitivity (nA/mM) of the ferrocene mediated glucose biosensor at different combinations of glucose oxidase loading and ferrocene loading ($n = 3$).

Glucose oxidase (mg/mL)	Ferrocene (M)			
	0	0.05	0.1	0.2
4.5	19.1 ± 1.3 (6.8%)	78.3 ± 1.8 (2.3%)	106.7 ± 6.3 (5.9%)	162.4 ± 3.5 (2.2%)
8.5	21.5 ± 0.5 (2.3%)	87.7 ± 4.3 (4.9%)	125.1 ± 3.6 (2.9%)	199.0 ± 5.7 (2.9%)
12.5	25.8 ± 1.7 (6.6%)	96.3 ± 1.0 (1.0%)	189.7 ± 1.3 (0.7%)	210.5 ± 4.9 (2.3%)
16.5	23.8 ± 0.1 (0.4%)	103.6 ± 0.1 (0.1%)	150.3 ± 10.4 (6.9%)	186.3 ± 6.0 (3.2%)

Relative standard deviations are stated in parentheses.

12.5 mg/mL glucose oxidase loading was found to be significantly higher than that of 4.5, 8.5, and 16.5 mg/mL glucose oxidase loading ($p < 0.05$). Interestingly, for biosensor with 0.2 M ferrocene, the sensitivity of 12.5 mg/mL glucose oxidase loading was found to be higher than that of 16.5 mg/mL glucose oxidase loading. It seemed that higher glucose loading doesn't take any advantage with higher concentration of ferrocene. Therefore, the optimum ferrocene mediated glucose biosensor in this study could be the combination of 12.5 mg/mL glucose oxidase with 0.1 M ferrocene and 12.5 mg/mL glucose oxidase with 0.2 M ferrocene. This finding was similar to that reported by Cass.[5]

The construction reproducibility of the ferrocene mediated glucose biosensor was evaluated from relative standard deviation of the slope of current response vs. glucose concentration plot (i.e., sensitivity). Between different biosensors of the same batch it was about 7% ($n = 3$) (see Table 2).

Drop-coating is a simple procedure on applying chemicals onto the surface of the transducer, but this must be balanced against the difficult of controlling the amount of chemicals deposited on the transducer. Therefore, in this study, a C-ring (10 mm thickness) was placed onto the surface of the transducer to form a boundary before dropping a known amount of chemical onto the surface of the transducer. This could greatly minimize the problem.

For amperometric biosensor, good results can be obtained by applying high oxidation potential. However, interferences from other electroactive species, such as uric acid, at high oxidation voltages can be found. In this study, as a result of the use of ferrocene, a low oxidation potential (200 mV) could be achieved and it was low enough to avoid excessive interference from other electroactive substances, but high enough to give easily measurable currents. Since uric acid can be transdermally extracted by reverse iontophoresis,[29] interferences can happen if uric acid is present and high oxidation potential is used. For the GlucoWatch®, the oxidation potential of its glucose biosensor is set to 420 mV[52,53] while the oxidation potential of our glucose biosensor was set to 200 mV. Since lower oxidation potential can reduce the interferences from other electroactive species, it can be expected that our glucose biosensor has less interference.

The glucose biosensor of the GlucoWatch® has a linear ($r^2 \sim 0.98$) glucose response range from 0 to 28 mM[52] which is larger than ours ($r^2 \sim 0.99$, 0–4 mM). However, the amounts of transdermal glucose extraction by reverse iontophoresis are several orders of magnitude lower than those present in the blood (~5 μM vs. ~5 mM).[52] Therefore, our glucose biosensor could cover the concentrations of the transdermally extracted glucose.

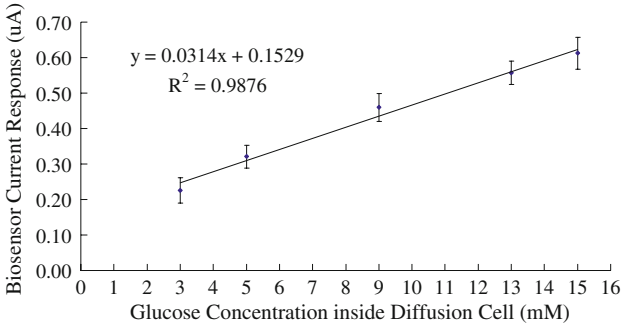

FIGURE 7. Evaluation of the ferrocene mediated glucose biosensor for reverse iontophoresis. An excellent linear relationship ($r^2 = 0.99$) between the biosensor current response and glucose concentration inside diffusion cell was found. Data ($n = 5$) was obtained from biosensor with the optimum combination of 12.5 mg/mL glucose oxidase and 0.1 M ferrocene. The cell was filled with an electrolyte solution comprising 0.1 M PBS pH 7.4 and 3–15 mM glucose.

Ferrocene Mediated Glucose Biosensor for Reverse Iontophoresis

The ferrocene mediated glucose biosensor coupled to our own developed reverse iontophoresis device[9] has been tested. The reverse iontophoresis device was used for trans-membrane (the membrane of the diffusion cell) glucose extraction. The extracted glucose was then quantified by the biosensor. The magnitude of the biosensor current response was plotted against the glucose concentration inside the diffusion cell (see Fig. 7). It was found that there was an excellent linear relationship ($r^2 = 0.99$) between the diffusion cell glucose concentration (3–15 mM) and biosensor current response. The reason for choosing 3, 5, and 15 mM glucose concentration was to simulate hypoglycemia, normal and hyperglycemia situation, respectively. Based on the results, our glucose biosensor was accurate enough on measuring glucose levels at both hypoglycemia and hyperglycemia. However, GlucoWatch® is unable to reliably detect hypoglycemia and hyperglycemia.[8]

CONCLUSION

A mediated glucose biosensor incorporated with reverse iontophoresis function for noninvasive glucose monitoring has been developed. The glucose biosensor exhibits a fast response, good sensitivity and reproducibility.

ACKNOWLEDGMENT

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