DEVELOPMENT OF A MICROCELL-BASED HYPER-SENSITIVE ASSAY SYSTEM FOR THE REVERSE IONTOPHORETICALLY EXTRACTED GLUCOSE

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Abstract: Human interstitial fluid glucose (ISFG) of a non-diabetic volunteer's skin was drawn transdermally by the reverse iontophoretic method. Concentrations of ISFG extracted by reverse iontophoresis at the cathodic extraction electrode were found to be extremely low in concentration as well as proportional to the blood glucose levels. In this study, the extracted ISFG sample were quantitatively analyzed by an amperometric method that utilized a high sensitivity flow type electrochemical cell to detect hydrogen peroxide; the product of the reaction between glucose and glucose oxidase(GOD). Also the pulsed amperometric detection (PAD) method was applied to overcome the difficulties in glucose detection under the condition that many interfering organic substances exist.

Introduction

Reverse iontophoretic glucose extraction is a promising technique for noninvasive, painless, and more frequent blood glucose monitoring[1-3]. With this technique, the extracted sample volume and its glucose concentration is too small to be analyzed with the conventional electrochemical analysis methods. One commercialized device based on reverse iontophoresis is still being used only for showing patterns and trends of changes in blood glucose level with some operational limitations [4]. One of the difficulties in developing and improving this type of glucose monitoring devices is the lack of an instrument to provide accurate reference data.

High performance liquid chromatography (HPLC) analysis or radioactivity measurement by a liquid scintillation counter may be possible candidates for the reference analysis[5]. However, in HPLC system, there exist high concentration of other electolytes which easily interfere the amperometric measurement. Radioactivity-based system is too complicated, too expensive and inadequate for personal use.

In this study, we proposed a microcell-based hypersensitive assay system to provide reference data for the extremely low level of glucose in a reverse iontophoretically extracted interstitial fluid sample.

Material and method

- ISFG extraction

Interstitial fluid glucose(ISFG) was extracted by a custom-made device which consists of a main control unit, user interface, and a constant current source. The extraction device was designed to adjust extraction parameters such as current level and time duration for extraction, through which we could find out the most effective extraction condition.

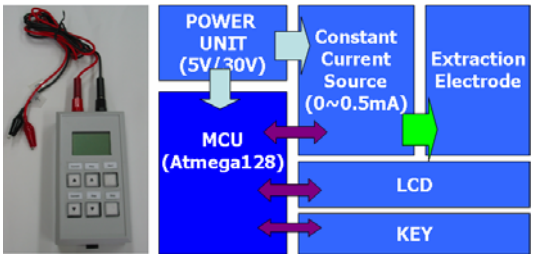

Figure 1: Picture of the extraction device and its functional block diagram

As extraction electrodes, a ring-shaped electrode (outer diameter 17mm; inner diameter 12mm) was printed using Ag/AgCl ink (Ercon Co.) on an epoxy resin plate (thickness 0.2mm) and annealed at 130℃ to remove volatile solvents. In order to make an extraction

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chamber filled with 600ul of extractant, a reservoir with 800 uL volume was prepared on the extraction electrode using an adhesive foam tape. Applying the extraction device to the inner skin of subject's forearm, a D.C. current with a range of 0.1~0.5 mA (current density 0.12~0.64 mA/cm2) was applied for 2~10 minutes for each reverse iontophoretic extraction experiment.

In order to minimize deterioration of the Ag/AgCl extraction electrodes, their polarities were alternated for each extraction. The extracted solutions at the cathode and the anode were withdrawn separately during a current-off phase of 20 minutes between adjacent extraction phases and stored in a refrigerator for further analysis.

- Microcell Electrode Fabrication and Instrumentation

In order to form a standard 3 electrode electrochemical microcell, thin film electrodes were fabricated on a glass substrate by the image reversal technique used in the photolithographic procedure.

For patterning of a photo-resistive(PR) layer, a glass substrate was spin-coated with HMDS at 4000 rpm for 30 sec and with AZ5214E at 4000 rpm for 30 sec and baked on a hot plate at 110ºC for 50 sec. And then it was exposed to an UV radiation through the mask film at 20mJ/cm^2 for 5.5 sec and baked at 125ºC for 2 min again. Removing the mask film, it was exposed at 20mJ/cm^2 for 12.5 sec. After developing for 65 sec, it was finally hard baked at 110ºC for 1 min. Thin layers of chrome(0.2μm) and platinum(1μm) were sputtered on to the surface of the PR patterned substrate. All patterned electrodes then has Pt surface. Working and counter electrodes were left as bare Pt electrodes as sputtered. Proprietary technology of fabricating a mesoporous Pt layer was used on the reference electrode's surface. Mesoporous Pt electrode had been shown to be a good micro-scale reference electrode[6] and made by the following recipe.

C8E16O (Octasethylene glycol monohexadecyl ether, Fluka) 0.42g, distilled water 0.29g and hydrogen hexachloroplatinate hydrate(Aldrich) 0.29g were mixed at 70 ºC. After mixed solution was totally degassed, the solution was cooled at room temperature (~26ºC) with a separate counter and reference electrodes for the plating operation in the mixed solution together. Electroplating proceeded at room temperature for 1800s referenced by a separate Ag/AgCl electrode.

A microcell with 1000μL volume was formed on to the fabricated thin film microelectrodes and completely sealed with two in/out tubes by a silicone glue. This electrochemical microcell was connected to a sample injector (9725i, Rheodyme, USA) which has sampling loop volume of 250μL powered by a peristaltic pump (52B073, Ismatec SA, Switzerland). Flow rate of eluent (pH 6.90 PBS solution) was 0.28 mL /min.

- Analysis method

The chemistry of this enzyme-catalyzed reaction is as follows;

$$\text{Glucose} + O2 \xrightarrow[\text{GOD}]{} \text{Gluconic Acid} + H_2O_2$$

H_2O_2 then produces a cathodic current at working electrode. According to the way of applying potential to an electrochemical cell, amperometric detection methods are categorized into the constant amperometric detection (CAD) or the pulsed amperometric detection (PAD).

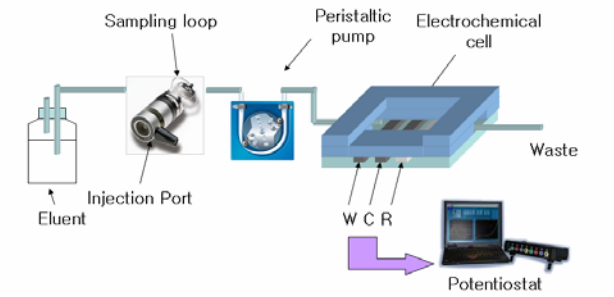

Figure 2: Block diagram of the developed microcell-based hypersensitive glucose assay system.

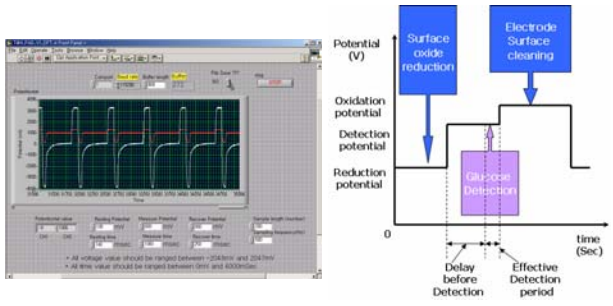

Figure 3: Screen display and potential waveform of the developed pulsed amperometric detection (PAD) system.

The CAD method applies a constant potential to the electrochemical cell but PAD applies the reduction and oxidation potential alternately between adjacent detection periods to cleanse the active electrode's surface. This alternating reduction and oxidation process is expected to suppress adsorption of various organic substances existing in the extracted sample.[7]

From a current-voltage curve of the microcell electrode and extensive optimizing tests of the developed PAD system, we successfully set the optimal parameters for the system. Reduction and oxidation potentials were -500mV for 300ms and 450mV for 150ms, respectively referenced by the mesoporous Pt electrode. Glucose detection signal was measured at 100mV for 300ms after a 200ms of time delay followed by a pair of reduction and oxidation process.

Amperometric detection unit was designed to have control functions to refine various conditions of reduction/oxidation potential and timing parameters which are closely related to performance of the PAD system.

- Experiments

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In-vitro performance of the developed system was verified with the solutions of different concentration (0.6, 1.25, 2.5, 5, and 10.0 ppm) of glucose in the presence of glucose oxydase(GOD). Same tests were repeated without GOD as a negative control experiment. Standard D-glucose (Aldrich) and glucose oxidase (Sigma, 47,200 units/g.) solutions were prepared in pH 6.90 PBS. Each glucose solution was prepared with phosphate buffer solution (PBS) at pH 6.9 from 0.1 M phosphate salts (Aldrich) and 77 mM sodium chloride (Aldrich) in de-ionized water.

For *in-vivo* performance tests, a 600uL sample of ISFG was repeatedly extracted with a 20~60 minutes time interval. To quantitatively analyze ISFG concentrations in the extracted aliquots, each extract was divided into two 250uL aliquots. One aliquot was mixed with 250uL PBS and the other was reacted with 250uL of GOD solution (50 Units/ml) for 6 minutes in order to generate hydrogen peroxide, the product of reaction between ISFG and glucose oxidase before being introduced to the flow type electrochemical sensing cell.

Results

- In-vitro performance

Results of the *in-vitro* performance tests are shown in Figure 4 and 5. Minimum detectable glucose concentration of the sample was 0.1 ppm. Magnitude of output responses in the cases of glucose standard solution without GOD and PBS with GOD was almost negligible.

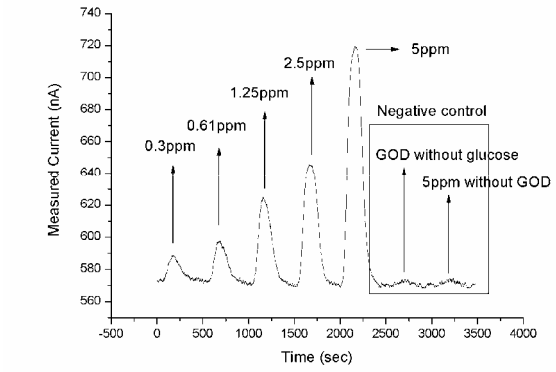

Figure 4: Result of *in-vitro* performance evaluation tests with 5 different concentrations of glucose solution and two negative control tests.

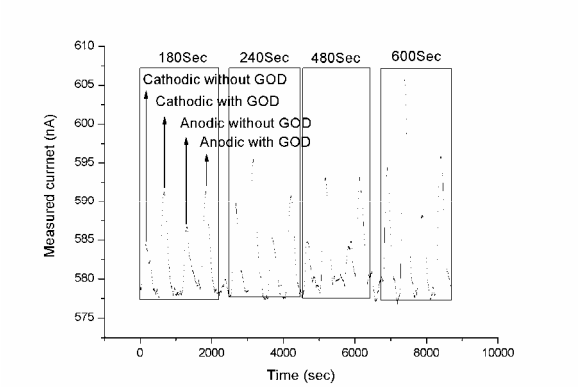

Figure 5: Result of *in-vitro* performance evaluation tests for the extracted samples with different iontophoretic current levels and different extraction time durations.

- In-vivo performance

As a preliminary human trial, body fluid samples were collected from a nondiabetic volunteer's forearm skin. Both anodic and cathodic extraction samples were separately analyzed by the developed system. For comparison, blood glucose level was measured with a conventional finger-pricking device at the end of each extraction period.

To optimize the extraction conditions, various parameters, such as extraction current level, electrode shape, extraction chamber size and time duration have been altered. Higher extraction current level and longer extraction time definitely produced higher output current signal. But for the prevention of skin irritations of stinging and itching feelings after extraction as well as Ag/AgCl electrode's deterioration, we determined to use the extraction chamber of 1cm in diameter and the current level of 0.2mA(0.38mA/cm^2) for 480sec.

Final glucose level was estimated by the output current level from the extraction sample and GOD mixture after subtracting the current level from the sample without GOD as a background correction. This subtracted value is exactly reflecting the glucose signal corresponding to H_2O_2 reduction current which produced by ISFG and GOD.

Figure 6 shows the result from the cathodic extraction sample analyzed by the CAD. Eleven cathodic samples were analyzed. Each extraction sample was halved and only one of them was mixed with GOD. In Fig. 6, the peaks marked with a primed number correspond to the sample with GOD. Despite of existence of unknown-origin negative peaks for each sample without GOD, the estimated glucose levels shows, in general, a trend toward the measured blood glucose levels in this case.

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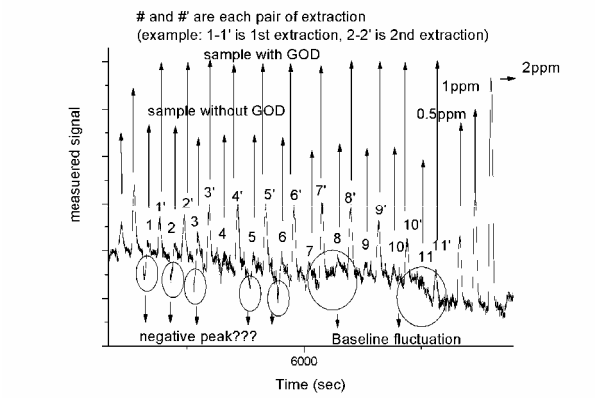

Figure 6: Result of *in-vivo* performance tests with the constant amperometric detection(CAD) method.

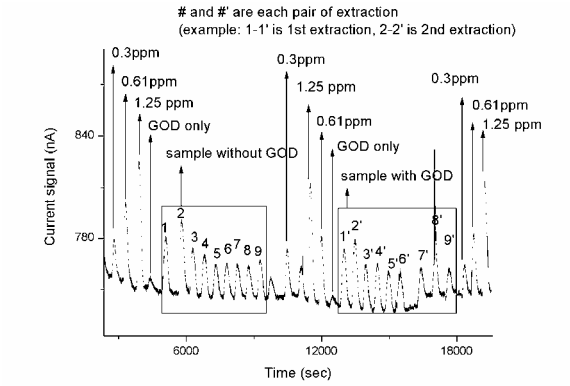

Figure 7: Result of *in-vivo* performance tests of anodic extraction with the pulsed amperometric detection (PAD) method.

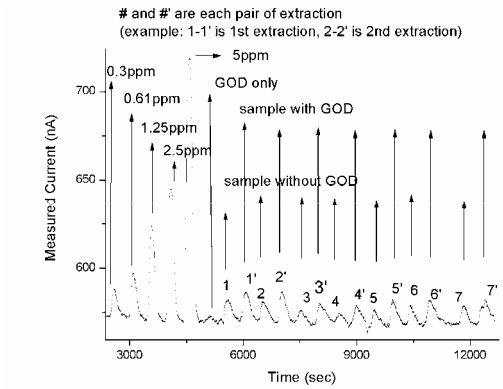

Figure 8: Result of *in-vivo* performance tests of cathodic extraction with the pulsed amperometric detection (PAD) method.

Figure 7 shows the results with anodic extraction samples analyzed by the PAD . Nine anodic samples were analyzed in the same way as in fig. 6. Unlike the case in Fig 6, there are no negative peaks at all. However, the correlation between the estimated glucose levels and the real blood glucose level was very low.

Figure 8 shows the results with seven cathodic extraction samples analyzed by the PAD in the exactly same way as Figs. 6 and 7. With the cathodic extraction samples analyzed by the PAD method, we could achieve highly correlated results with the real blood glucose levels.

Both figure. 9 and table 1 show a well-matched result of this case.

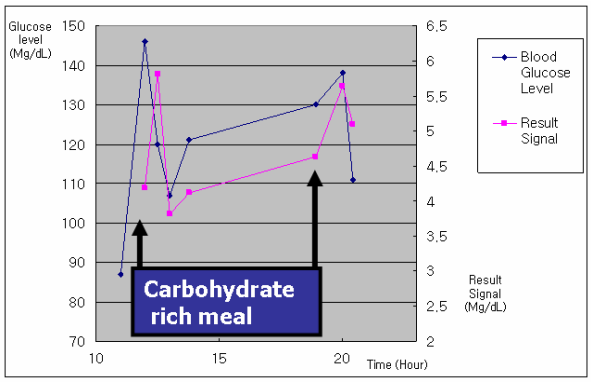

Figure 9: Estimated glucose signal for cathodic extraction case in comparison with the real blood glucose level measured by finger-pricking strip sensors.

Table 1: Results of the preliminary human trial

Time (Hr:Min)	Blood glucose level (mg/dL)	Result signal
AM 11:00	87	N/A
AM 12:10	146	4.181
AM 12:30	120	5.818
PM 1:00	107	3.818
PM 1:40	121	4.120
PM 6:15	130	4.640
PM 7:50	138	5.636
PM 8:20	111	5.100

Discussions and Conclusions

A microcell-based hyper sensitive glucose assay system was developed for the purpose of providing reference data for the determination of the reverse iontophoretically extracted interstitial fluid glucose level. From *in-vitro* performance tests using standard glucose solutions, the developed system provided a very high correlation coefficient of 0.99. Also, in *in-vivo* tests, measured signal showed a high correlation with the real blood glucose level except only one case, the very first one.

As we have experienced in many previous experiments, the CAD generated many unknown negative peaks and baseline wandering. Compared with the CAD, the PAD provided more stable output signals as well as less baseline fluctuations. But overall glucose signal level in the PAD was slightly weaker than that in the CAD, which is thought to be caused by of the fact that reduction of H_2O_2 in the PAD occurs not only in detection period but also in reduction period. For both methods of amperometric detection, there existed substantial signal level for the sample without GOD. These signal components possibly caused by the interfering substances such as ascorbic acid is expected

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to be successfully suppressed by coating electrodes with an outer membrane using Nafion.

In this preliminary study, we were able to quantify the concentrations of ISFG using a relatively simple flow type electrochemical cell consisting of a platinum working and counter electrodes and the proprietary mesoporous platinum reference electrode. The sensitivity of the developed system was found to be high enough to detect extremely low level of the extracted ISFG. Also, applying the PAD to analysis method, we achieved more stable results. Overall results imply that the developed technology will be promisingly applied to real clinical situations as a minimally invasive glucose measurement technique.

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