whereas larger interfering molecules, such as uric acid and acetaminophen, are significantly attenuated, resulting in an enhanced selectivity of the H$_2$O$_2$ (from enzymatic oxidation of glucose) response at the sensor surface.

We examined the effectiveness of the hydrogel-assembly system by depositing known concentrations of model compounds on the hydrogels in the presence and absence of the phenolic compounds and measuring the responses of the underlying Pt/C sensor to the compounds. As model compounds, we studied glucose (200 μmol/L), uric acid (100 μmol/L), acetaminophen (230 μmol/L), and others (not shown). The concentrations of the target and interfering compounds were based on the flux of 3 mmol/L blood glucose through intact skin (6). For a flux rate of 1 nmol/h·cm$^2$ and a transdermal patch area of 2.85 cm$^2$, the skin extract was estimated to contain 0.713 nmol of glucose and an equivalent concentration of acetaminophen. For convenience and reliability of benchtop measurements, the concentrations of the interfering compounds found in the extract were ratioed to that of glucose to give the values stated above. Before depositing the compounds on the hydrogels, the sensor system was preconditioned sequentially, at 0.77 V vs Ag/AgCl for 10 min and at 0.42 V vs Ag/AgCl for 50 min. After the preconditioning sequence, the test compounds were added to the hydrogel, and the resulting currents were measured and integrated. The resulting charges were used for analysis.

Fig. 1 shows time-dependent responses of hydrogel-sensor systems in the presence and absence of phenolic additives in the hydrogel to the test compounds. The assemblies containing standard hydrogel showed the greatest sensitivity to acetaminophen, followed by uric acid and glucose. However, in hydrogel with phenolic additive, the trend was reversed within 10 min of measurement. Each hydrogel-sensor assembly was tested once, and at least six assemblies were used to test each compound. The test method had a CV (n = 12) of 4.5%.

As expected, the in situ membrane film formed at the hydrogel-sensor interface during the preconditioning period attenuated the response of the system to uric acid and acetaminophen, whereas the impact on H$_2$O$_2$ (i.e., the glucose signal) was insignificant. For instance, compared with the assembly containing standard hydrogels, the responses of the sensor system containing phenolic hydrogel to uric acid and acetaminophen at 5 min were reduced by 65% and 79%, respectively, whereas the reduction for glucose was ~23%. For example, the responses of the assembly to acetaminophen in the absence and presence of the phenolic compound were 329,088 ± 13,419 and 67,728 ± 6642 nC, respectively, a 79% decrease in sensitivity to acetaminophen.

References


Design of a Biosensor for Continual, Transdermal Glucose Monitoring, Michael J. Tierney,* Yalia Jayalakshmi, Norman A. Parris, Michael P. Reidy, Christopher Uhegbu, and Prema Vijayakumar (Cygnus, Inc., Redwood City, CA 94063; * author for correspondence: fax 650-369-5318, e-mail Tierney@cygn.com)

The Diabetes Control and Complications Trial (1) and other trials have shown that frequent monitoring of blood glucose by diabetics is critical to achieving normoglycemic concentrations. The ability to achieve this goal is hampered, however, by the lack of any means of obtaining frequent blood glucose data in a convenient and unobtrusive manner.

To address these needs, a continual, noninvasive glucose monitoring system has been developed that utilizes reverse iontophoresis to induce an electroosmotic flux of glucose through intact skin. The electroosmotic glucose flux (measured ex vivo by HPLC) was shown in earlier clinical trials to correlate with the blood glucose concentrations (2). To create a miniaturized and wearable system, an amperometric biosensor has been developed that measures in situ the glucose extracted through the skin into a hydrogel pad to provide periodic measures of blood glucose every 20 min over the course of 12 h. A diagram of this system is shown in Fig. 1A.

A major advancement that has been achieved in the development of this biosensor is the ability to accurately measure the small amounts of glucose that are extracted through the skin. These glucose concentrations are several orders of magnitude lower than those present in the blood (~5 μmol/L vs ~5 mmol/L), representing a total amount of glucose in the 50–200 picomol range. The main design criteria are summarized below.

Low concentration of glucose. The small amount of glucose extracted requires a biosensor with a detection limit much lower than conventional devices attain. A low detection limit has been achieved through the use of a large surface area working electrode and the development of a screen-printable platinum-graphite composite electrode material with high sensitivity and low background current. The large electrode, coupled with coulometric measurements, allows detection of a large fraction of the glucose collected into the hydrogel pad every cycle.

The characteristics of the biosensor were investigated in an in vitro cadaver skin diffusion cell thermostated at 32 °C. This cell consists of a donor solution compartment separated from the biosensor/iontophoresis assembly by...
a layer of heat-separated human epidermis. This test mimics very closely the in vivo performance of both the iontophoresis and biosensor functions. In this test, the integrated biosensor current responded linearly when the glucose concentration in the donor solution was increased stepwise through the range from 0 to 5000 mg/L (0 to 500 mg/dL) glucose [mean correlation coefficient (r) over six separate systems, 0.992; range, 0.965–0.999].

**Continual measurements.** The continual, periodic measurement cycle requires that the sensing be completed and that the previously extracted glucose be depleted for the next iontophoresis cycle to begin anew. Each measurement cycle is made up of two half-cycles, each consisting of 3 min of iontophoresis and 7 min of biosensing. The polarity of the iontophoresic current alternates for each half-cycle. The sensor geometry, diffusion distances through the hydrogel pad, glucose oxidase enzyme kinetics, glucose mutarotation kinetics, and electron transfer kinetics on the electrode all affect the dynamics of the measurement cycle. These parameters have been co-optimized using computer modeling (3) to achieve high sensitivity glucose measurement and subsequent depletion of remaining glucose within the required time period.

The sensitivity of the system must be stable to make repeated measurements over 12 h. Stability was investigated in nine separate in vitro diffusion cell systems. Each system was calibrated at the beginning of the measurement period to account for skin flux differences and the usual variances in the sensitivity of the biosensor. Three systems each were stepped to glucose concentrations of 500, 1000, and 4000 mg/L (50, 100, and 400 mg/dL), and the stability was evaluated over 36 measurements over the course of 12 h (Fig. 1B). The stability, as measured by the mean CV for the three cells at each concentration was 7.7%, 5.2%, and 5.1% for 500, 1000, and 4000 mg/L concentrations, respectively.

**Selectivity to glucose.** The biosensor has low sensitivity to most potential interfering species. This selectivity was achieved by utilizing the inherent permselective properties of the skin and the electroosmotic extraction process. The extraction through skin provides both size- and charge-exclusion properties. The size-exclusion properties arise from the molecular weight cutoff of ~500 for efficient extraction of compounds through the skin (4). The charge-exclusion properties are caused by the net negative charge carried by skin at physiological pH (5). Because of this charge, the electroosmotic flux, which carries the glucose, flows predominantly toward the iontophoretic cathode. Anionic species, such as the interfering species ascorbate and urate, migrate solely to the iontophoretic anode. By sensing glucose only at the biosensor at the iontophoretic cathode, the biosensor avoids interference from these species. Finally, by integrating the biosensor signal over the measurement cycle, the biosensor can operate at a fairly low potential (0.42 V vs a Ag/AgCl electrode). This potential is much lower than is typically used in glucose sensing and is at a value where some interfering species no longer react at the electrode. For example, background currents from tyrosine and tryptophan, which are seen at 0.6 V, are completely eliminated at 0.42 V.

**Toxicity.** Because the device is worn on the skin, the iontophoretic process would effectively deliver any mobile chemical species into the skin. This requirement precludes the use of sensing chemistries containing soluble mediators and other schemes incorporating mobile toxic or irritating compounds. The sensing chemistry and all other components of the device must pass strict skin toxicity and irritation tests.

The GlucoWatch® biographer (developed by Cygnus, Inc., Redwood City, CA) embodies the electroosmotic extraction and biosensor system described above in a...
small, wristwatch device. This device has been tested extensively in clinical trials on diabetic subjects and has been shown to accurately measure blood glucose in a continual and noninvasive manner.

References

Development of Kinetic Ligand-binding Assays Using a Fiber Optic Sensor, Richard H. Smith, William J. Lemon, Judith L. Erb, John R. Erb-Downward, James G. Downward, Otho E. Ulrich, and James L. Wittliff

Ligand-binding assays are ubiquitous in biochemical research and clinical determinations. In most common assay methods, binding proceeds until an equilibrium condition has been obtained, which leads to relatively long incubation times. The requirement for separating bound from free reagents in the reaction mixture before signal detection precludes direct observation of binding events. To reduce the duration of an assay and to facilitate kinetic analysis, methods based on evanescent field technology have recently been used in assay development. These include fiber optic fluorometric sensors (1–3) and surface plasmon resonance (4). Specifically, evanescent field technology is based on the observation that when light travels through a waveguide at angles approaching the critical angle for total internal reflection, an evanescent field is produced on the surface of the waveguide. This field falls off exponentially with distance from the surface and is exquisitely sensitive to the refractive indices of the waveguide surface and the medium in which the surface resides. In surface plasmon resonance, this sensitivity to refractive index is used to measure the amount of substance that binds to the waveguide surface. In evanescent field fluorometry, the field stimulates fluorophores, which become attached to the surface through specific binding interactions (2). We describe here the use of evanescent fiber optic fluorometric sensors to characterize ligand binding of IgG and monovalent Fab, both specific for estrone-1-glucuronide (E1g), and of the human estrogen receptor α (hER-α) to fibers bearing E1g or the specific estrogen response element (ERE) and demonstrate the determination of apparent association and dissociation binding constants.

Fused silica optical fibers obtained from Polymicro Technologies were cleaved in 11.5-cm lengths, and the cladding was removed from a 7.0-cm portion of the fiber using FluorinertTM (3 M). To assess the binding kinetics of IgG, Fab, and hER-α, fibers were sensitized by a modification of the method of Bhatia et al. (5). Reagents were obtained from Sigma-Aldrich unless otherwise noted. Fibers were placed in a 20 mL/L solution of 3-((mercapto)propyl)trimethoxysilane in dry toluene for 2 h at room temperature, and then rinsed in toluene, creating a glass surface bearing thiol groups. Thiols were reacted with the heterobifunctional agent, γ-maleimidobutyric acid-N-hydroxysuccinimide ester, 2 mmol/L in reagent alcohol for 1 h. To make E1g fibers, the succinimide ester was reacted for 1 h with 0.05 g/L casein in 20 mmol/L carbonate, pH 9.3. To prepare ERE fibers, 4 mg/L 5′-amino-ERE (nucleotide sequence, 5′ GTCCAAAGTCAGCTCACTGAGCCTGATCAAAGTT 3′; Research Genetics) was substituted for the protein. E1g was activated using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and then incubated with casein-derivatized fibers for 1 h. After extensive washing, fibers were air-dried and stored under dessicator. For binding assays, fibers were mounted inside capillary tubes having a 1.1-mm inner diameter and 80 μL final volume, and ferrules were applied to the ends to permit sample injection and continuous fluid flow through the reaction cell.

Monoclonal antibody specific for E1g was obtained from Dr. Fortune Kohen (Weizmann Institute, Rehovat, Israel). Fab was prepared by papain digestion followed by purification using protein G to remove unreacted IgG and IgG fragments. hER-α was prepared as described by Wittliff et al. (6). Proteins were labeled with Cy5 (1,3,3,3′,3′,1′,1′-heptamethyl-indodicarbocyanine sulfonic acid; Amersham) according to manufacturer’s instructions. IgG was used at a final concentration of 2 × 10−8 mol/L, and Fab was used at a final concentration of 4 × 10−8 mol/L. The concentration and affinity of hER-α were estimated by radioligand-binding assay (7), and Cy5-labeled hER-α was used in fiber optic assays at 5 × 10−10 mol/L. Binding of fluorophore-labeled protein to coated fibers was determined using an evanescent fiber optic fluorometer (Threefold Sensors) (8). To maximize the strength of the evanescent field, the fluorometer directed light from a 637 ± 2 nm laser diode into the fiber optic sensor cartridge at an angle just less than the critical angle. A holographic band-stop filter centered at 637 nm allowed the Stokes-shifted fluorescence emitted from Cy5 at wavelengths longer than 650 nm to pass with high efficiency. Light was detected by a photodiode, and the resulting currents were measured by a lock-in amplifier (model SR810; Stanford Research Systems) and collected on a computer using a program written in Labview (National Instruments).

To determine rate constants for association and dissociation, kinetic data were fitted to a mathematical model...