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An Amperometric Fructose Biosensor Based on Fructose Dehydrogenase Immobilized in a Membrane Mimetic Layer on Gold

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A prototype amperometric fructose biosensor based on membrane-bound fructose dehydrogenase *(Gluconobacter* sp.*)* and the coenzyme ubiquinone-6 immobilized in a membrane mimetic layer on a gold electrode has been constructed and tested. A bare gold electrode first was modified through chemisorption of a mixture of octadecyl mercaptan and two short-chain disulfides, 3,3′-dithiodipropionic acid and cystamine dihydrochloride. The membrane-bound enzyme, coenzyme, and additional phospholipid were codeposited through a detergent dialysis protocol. The short-chain modifiers may provide electrostatic interactions with enzyme surface charges, while the alkanethiolate and phospholipids enable hydrophobic interaction with the largely lipophilic, membrane-bound enzyme. At oxidizing potentials, the enzyme electrode responded with catalytic current densities up to 45 μA/cm^2 when exposed to fructose at 10 mM. The sensor exhibited a response time of less than 20 s, a sensitivity of 15 μA/cm^2·mM and a detection limit of less than 10 μM. Biosensor measurements of D-fructose in apple and orange juice agreed to within a few percent with those made with an enzymatic spectrophotometric assay. The membrane mimetic layer effectively blocked access of interfering ascorbic acid to the electrode surface. Only a 4% positive error was observed in the presence of ascorbic acid at 5% of the fructose concentration (2 mM), which indicates that this construct could be particularly useful for quantitation of fructose in citrus juice.

Reliable fructose sensors could be of value for quantitation of the sugar in food products such as fruit juice, high-fructose corn syrup, and wine, as well as in clinical samples including blood serum and seminal plasma. An enzymatic spectrophotometric assay is available for fructose determination; however it is time intensive, tedious, and costly.[1] Several groups recently have described the immobilization of *Gluconobacter* sp. fructose dehydrogenase (EC 1.1.99.11), a 140 kDa membrane-bound pyrroloquinoline quinone-containing oxidoreductase, on various electrodes to give potential fructose biosensors. Aizawa and coworkers immobilized the enzyme on glassy carbon, gold, and platinum, entrapped it in conductive polypyrrole matrices on platinum, and coupled it with the organic conducting salt TTF-TCNQ in a polypyrrole matrix on glassy carbon.[2−4] Alternatively, the enzyme has been secured on a carbon paste electrode behind a dialysis membrane with and without the mediator benzoquinone.[5,6] While these approaches delivered promising results in terms of current density, response time, and stability, readily oxidizable inteferents such as ascorbic acid in citrus juice have been found to overwhelm fructose signals.[5,6]

Immobilization of the membrane-bound enzyme in a lipophilic, self-assembled alkanethiolate layer on a gold electrode might provide an effective solution to this problem. Alkanethiols, such as octadecyl mercaptan, chemisorb quite readily to gold forming well-ordered, densely packed monolayers.[7−9] In addition to providing a membranelike environment for the enzyme, the hydrophobic monolayer can reduce access of polar electroactive species to the electrode surface.[7,10−13] Others have constructed glucose biosensors using the perfluorinated polyanionic polymer Nafion as a barrier to electroactive anions, including ascorbate.[14,15] An alkanethiolate layer may have the advantage of effectively blocking all, not just anionic, electroactive polar species from nonspecific interaction with the electrode surface. Immobilization and electronic coupling of a membrane-bound enzyme in a thiolate layer on gold was demonstrated previously for both fructose dehydrogenase (FDH)[16] and *Escherichia coli* fumarate reductase.[17] Fumarate reductase was immobilized in a chemisorbed octadecyl and dodecyl mercaptan monolayer on a gold electrode, and direct electron transfer between the electrode and the redox

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(1) Nakashima, K.; Takei, H.; Adachi, O.; Shinagawa, E.; Ameyama, M. *Clin. Chim. Acta* **1985**, *151*, 307−310.
(2) Khan, G. F.; Shinohara, H.; Ikariyama, Y.; Aizawa, M. *J. Electroanal. Chem.* **1991**, *315*, 263−273.
(3) Khan, G. F.; Kobatake, E.; Shinohara, H.; Ikariyama, Y.; Aizawa, M. *Anal. Chem.* **1992**, *64*, 1254−1258.
(4) Begum, A.; Kobatake, E.; Suzawa, T.; Ikariyama, Y.; Aizawa, M. *Anal. Chim. Acta* **1993**, *280*, 31−36.
(5) Ikeda, T.; Matsushita, F.; Senda, M. *Agric. Biol. Chem.* **1990**, *54*, 2919−2924.
(6) Ikeda, T.; Matsushita, F.; Senda, M. *Biosens. Bioelectron.* **1991**, *6*, 299−304.
(7) Porter, M. D.; Bright, T. B.; Allara, D. L.; Chidsey, C. E. D. *J. Am. Chem. Soc.* **1987**, *109*, 3559−3568.
(8) Sabatani, E.; Rubinstein, I.; Maoz, R.; Sagiv, J. *J. Electroanal. Chem.* **1987**, *219*, 365−371.
(9) Widrig, C. A.; Chung, C.; Porter, M. D. *J. Electroanal. Chem.* **1991**, *310*, 335−359.
(10) Creager, S. E.; Collard, D. M.; Fox, M. A. *Langmuir* **1990**, *6*, 1617−1620.
(11) Bilewicz, R.; Majda, M. *Langmuir* **1991**, *7*, 2794−2802.
(12) Takehara, K.; Ide, Y. *Bioelectrochem. Bioenerg.* **1992**, *27*, 207−219.
(13) Chailapakul, O.; Crooks, R. M. *Langmuir* **1993**, *9*, 884−888.
(14) Andrieux, C. P.; Audebert, P.; Divisia-Blohorn, B.; Linquette-Maillet, S. *J. Electroanal. Chem.* **1993**, *353*, 289−296.
(15) Mizutani, F.; Yabuki, S.; Katsura, T. *Anal. Chim. Acta* **1993**, *274*, 201−207.
(16) Kinnear, K. T.; Monbouquette, H. G. In *Biosensor and Chemical Sensor Technology: Process Monitoring and Control*; Rogers, K. R., Mulchandani, A., Zhou, W., Eds.; ACS Symposium Series 613; Washington, DC, 1995; pp 82−86.
(17) Kinnear, K. T.; Monbouquette, H. G. *Langmuir* **1993**, *9*, 2255−2257.

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centers of the enzyme was established. For FDH, however, the coadsorption of short-chain, functionalized disulfides, which form $SCH_2CH_2CH_2COO^-$ and $SCH_2CH_2NH_3^+$ on the electrode surface,[18] was required for electrostatic binding of the enzyme. Also, a lipophilic mediator, ubiquinone-6 (coenzyme Q-6), was needed to couple electronically FDH to the electrode. In this earlier work, it was found that loss of Q-6/FDH electrode activity was due primarily to coenzyme desorption from the electrode.[16]

We describe in this paper a method to improve both stability of the coenzyme on the electrode surface and ascorbic acid rejection. Our method is related to the work of Cullison et al.,[19,20] where the enzyme cytochrome *c* oxidase is immobilized in a phospholipid bilayer anchored to a submonolayer, octadecyl mercaptan-modified gold electrode.[21] In our work, the enzyme and coenzyme are immobilized with phospholipid on a gold electrode modified with a mixture of octadecyl mercaptan and two shorter chain, charged disulfides. The addition of phospholipid to the enzyme electrode construct not only improves coenzyme retention and enzyme stability but also provides added insulation against electroactive interferents such as ascorbic acid. In this paper, we also report Q-6/FDH electrode characteristics such as response time and sensitivity as well as the performance of the electrode as a sensor for fructose in fruit juice.

EXPERIMENTAL SECTION

Reagents and Materials. D-Fructose dehydrogenase from *Gluconobacter* sp. (FDH), coenzyme Q-6, D(−)fructose, and *n*-octyl *β*-D-glucopyranoside (*n*-octyl glucoside) were purchased from Sigma and used without further purification. Octadecyl mercaptan, 1,4-benzoquinone, cystamine dihydrochloride and 3,3′-dithiodipropionic acid were from Aldrich. The Test Combination D-glucose/D-Fructose assay kit was obtained from Boehringer Mannheim. Dioleoyl-L-phosphatidylethanolamine (DOPE) and dioleoyl-L-phosphatidylcholine (DOPC), in chloroform, were purchased from Avanti Polar Lipids, Inc. Hexaammineruthenium(III) chloride was obtained from Strem Chemicals (Newburyport, MA). Fisher or Sigma served as general sources of other reagent grade chemicals. All electrodes were purchased from Bioanalytical Systems, Inc. (W. Lafayette, IN).

Electrochemical Measurements. Electrochemical measurements were made with an Omni 90 potentiostat (Cypress Systems, Inc.). The Omni 90 was interfaced to a Macintosh IIcx with a National Instruments (Austin, TX) Lab-NB board and software (LabVIEW II). Gold disk electrodes (d = 1.6 mm, A_{geom} = 0.02 cm^2), a Ag/AgCl (3 M NaCl) electrode, and a platinum wire were used as the working, reference, and counter electrode, respectively. Blocking of methyl viologen, $Fe(CN)_6^{3-}$, benzoquinone, and $Ru(NH_3)_6^{3+}$ from the electrode surface was studied through cyclic voltammetry of 5 mM solutions of each species in 0.1 M NaCl at a scan rate of 50 mV/s. Amperometric measurements of fructose and other sugars in the presence or absence of ascorbic acid were done at 0.5 V vs Ag/AgCl in stirred deoxygenated 10 mM KH_2PO_4, pH 4.5, under a blanket of argon at room temperature unless otherwise stated.

Modified Electrode Preparation. Prior to phospholipid deposition and enzyme immobilization, freshly polished and electrochemically cleaned electrodes[17] were modified for 2 h in 40 mol % octadecyl mercaptan, 30% cystamine dihydrochloride, and 30% 3,3′-dithiodipropionic acid ethanol solutions. The total thiol/disulfide concentration was 1 mM. The disulfides undergo dissociative adsorption at the gold surface and form self-assembled monolayers in much the same way as alkanethiols.[22]

Immobilized Enzyme/Coenzyme/Lipid Preparation. Approximately 1 mL of DOPE and 0.24 mL of DOPC (both 25 mg/mL) were added to a round-bottom flask that contained 100 mg of octyl glucoside and 300 μL of 1.7 mM coenzyme Q-6 (final Q-6 concentration 170 μM). The solvent was removed by evaporation under vacuum (35 °C, 5 h). Cold phosphate buffer (3 mL), pH 4.5, was added to the flask, and the solution was stirred overnight at 4 °C. The clear solution was then filtered through a 1.2 μm filter to remove any particulates. FDH (3 mg) was added to the filtrate; the solution was stirred for 15 min and then allowed to equilibrate for 2 h at 4 °C. The enzyme solution (300 μL) and the previously modified electrode were added to a 10 000 MWCO dialysis bag, rinsed and soaked in water prior to use, and placed in a 400 mL reservoir of phosphate buffer; dialysis was done at 4 °C under stirring conditions for 2−6 days. The dialysate was replaced four to six times; the last reservoir replacement contained ∼1 mL of the detergent-sorbing resin, Calbiosorb. The electrode was removed from the bag and rinsed with Millipore water and either used immediately or stored in buffer.

Determination of Enzyme Coverage. The amount of enzyme on the gold electrode was determined spectrophotometrically according to the method of Ameyama et al.[23] In order to determine enzyme coverage only on the gold surface, the plastic surrounding the gold disk was removed. To remove the enzyme from the lipid/alkanethiolate layer, the gold electrode tip was placed in 0.1% Triton X-100 and gently agitated for 1.5 h. The detergent solution was then assayed for FDH activity.

RESULTS AND DISCUSSION

A comparison of cyclic voltammograms of 5 mM methyl viologen, $Fe(CN)_6^{3-}$, benzoquinone, and $Ru(NH_3)_6^{3+}$ solutions conducted with bare electrodes and electrodes modified with adsorbed thiols and phospholipids (no enzyme) illustrates the variable blocking characteristics of the modified electrode system (see Figure 1). The oxidation peaks for negatively charged ferricyanide and neutral benzoquinone were reduced substantially by 88 and 80% relative to the bare electrode, and the peak distortion and increased voltage separation indicated that the redox reactions were limited to a small number of sites on the electrode surface. However, rejection of positively charged hexaammineruthenium and methyl viologen was reproducibly less impressive; the cyclic voltammograms retain at least quasi-reversible character, suggesting that these molecules still can react at a substantial fraction of the modified electrode surface. These results are in qualitative agreement with literature data on the blocking of electroactive species from gold electrode surfaces modified with chemisorbed octadecyl mercaptan (OM), although the overall insulating properties of the mixed thiolate/phospholipid layer are not as good. OM monolayers exhibited nearly complete

(18) Hill, H. A. O.; Lawrance, G. A. *J. Electroanal. Chem.* **1989**, *270*, 309−318.
(19) Cullison, J. K.; Hawkridge, F. M.; Nakashima, N.; Hartzell, C. R. in *Charge and Field Effects in Biosystems;* Allen, M. J., Cleary, S. F., Sowers, A. E., Shillady, D. D., Eds.; Birkhäuser: Boston, 1992; Vol. 3, pp 29−40.
(20) Cullison, J. K.; Hawkridge, F. M.; Nakashima, N.; Yoshikawa, S. *Langmuir* **1994**, *10*, 877−882.
(21) Plant, A. L. *Langmuir* **1993**, *9*, 2764−2767.
(22) Nuzzo, R. G.; Fusco, F. A.; Allara, D. L. *J. Am. Chem. Soc.* **1987**, *109*, 2358−2368.
(23) Ameyama, M.; Shinagawa, E.; Matsushita, K.; Adachi, O. *J. Bacteriol.* **1981**, *145*, 814−823.

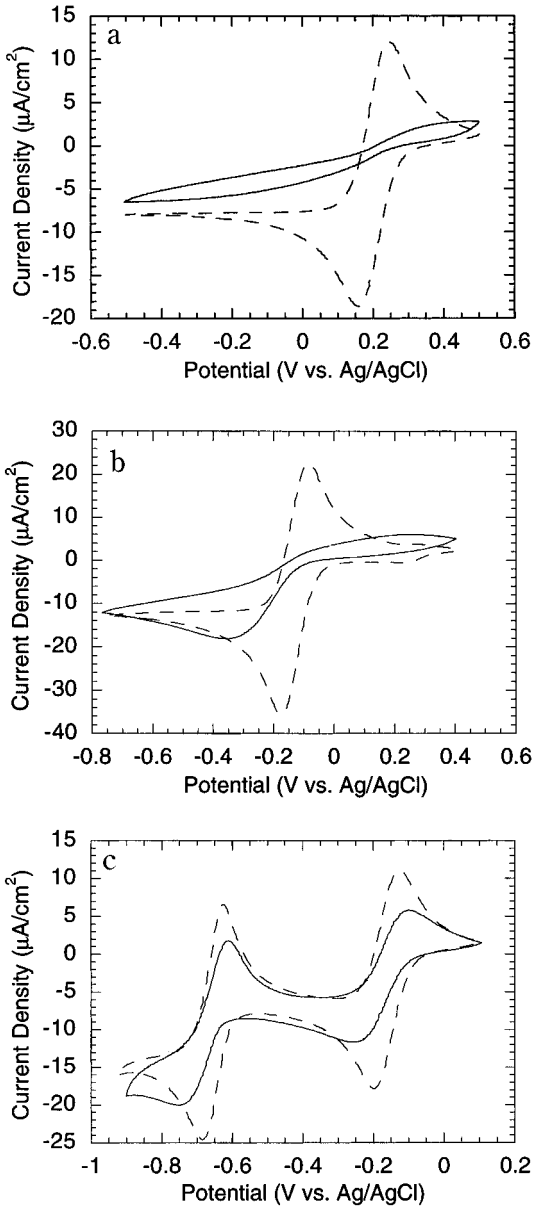

Figure 1. Steady-state cyclic voltammograms of bare (dashed curves) and phospholipid/thiolate-modified (solid curves) electrodes in solutions of 100 mM NaCl and 5 mM (a) $Fe(CN)_6^{3-}$, (b) 1,4-benzoquinone, and (c) methyl viologen and $Ru(NH_3)_6^{3+}$. The scan rate was 50 mV/s.

blocking of $Fe(CN)_6^{3-}$ from the surface,[7,10,13] yet measurable electroactivity of $Ru(NH_3)_6^{3+}$,[10,11,13] methyl viologen,[10] and a derivative of benzoquinone, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (ubiquinone-0),[12] was observed. It may be that the negative, carboxyl-terminated ligands present on our electrode preparations enhance the electroactivity of the positively charged reagents. Nevertheless, our results with $Fe(CN)_6^{3-}$, in particular, were encouraging since the most important electroactive interfering agent in fruit juice is negatively charged ascorbate.

When coenzyme Q-6 and FDH were coimmobilized in the mixed thiolate/phospholipid layer on gold, electroenzymatic activity was evidenced by comparison of cyclic voltammograms obtained in the absence and presence of fructose as shown in Figure 2. In buffer, the redox waves of coenzyme Q-6 were visible; however, in the presence of 20 mM fructose, a significant stable catalytic current was observed due to oxidation of fructose by FDH and turnover of coenzyme Q-6 by the electrode. To confirm the

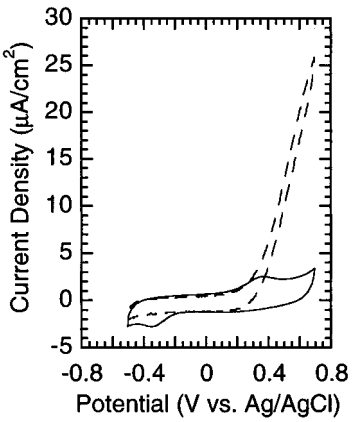

Figure 2. Steady-state cyclic voltammograms of Q-6/FDH electrode in deoxygenated, 10 mM KH_2PO_4, pH 4.5 buffer (solid curve) and with 20 mM fructose added (dashed curve). The scan rate was 50 mV/s.

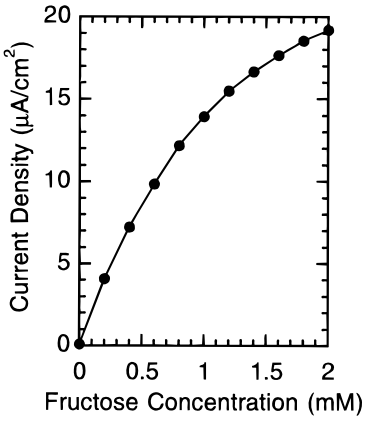

Figure 3. Calibration curve for D-fructose determined by a Q-6/FDH electrode.

role of the enzyme in the catalytic response to fructose, Q-6 was immobilized in a mixed monolayer electrode without FDH. This electrode did not respond catalytically when exposed to fructose. In additional control experiments, modified electrodes with immobilized FDH and no coenzyme, as well as bare Au electrodes subjected to the dialysis procedure in the absence of enzyme, did not exhibit Faradaic current in buffer or fructose.

Figure 3 depicts a typical calibration curve obtained with a Q-6/FDH modified electrode poised at a potential of 0.5 V vs Ag/AgCl where reoxidation of Q-6 by the electrode is assured. At lower concentrations of fructose, a linear relationship between current and concentration was observed, while current approaches a saturation value at higher fructose concentrations (data not shown). Due to surface irregularities, electrode responses were variable from electrode to electrode. However, all electrodes followed similar trends. The typical calibration curve was linear to ~0.5 mM. The sensitivity, or the slope, of the plot in Figure 3 was 15 $\mu A/cm^2 \cdot mM$. Sensitivity values ranged from about 10 to 20 $\mu A/cm^2 \cdot mM$. Current densities (four electrodes) at 10 mM fructose ranged from 12 to 45 $\mu A/cm^2$. At 1 mM fructose, current densities (seven electrodes) varied from 3 to 15 $\mu A/cm^2$. The lowest fructose concentration measured was 10 μM; however, the detection limit with the 0.02 cm^2 electrode could be as low as 250 nM at a current of 1 nA based on extrapolation from the calibration curve. The relative standard deviation for five successive measurements of 4 mM fructose obtained with a Q-6/FDH electrode was 2.4%.

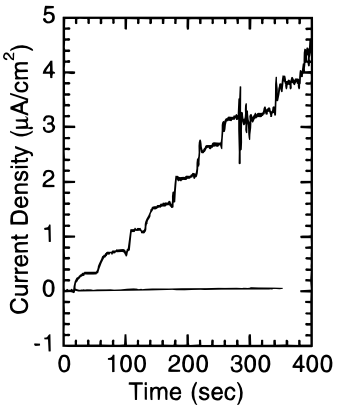

Figure 4. Current response of a bare (upper curve) and a Q-6/FDH (lower curve) electrode at 0.5 V vs Ag/AgCl to ascorbic acid from 0 to 1.0 mM added in 0.1 mM increments. Solution conditions: magnetically stirred, 10 mM KH_2PO_4, pH 4.5.

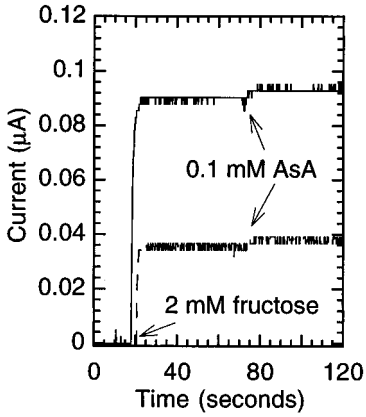

Figure 5. Response of a Q-6/FDH electrode to injected fructose and ascorbic acid over 22 days; day 6 (solid curve) and day 28 (dashed curve). The electrode potential was poised at 0.5 V vs Ag/AgCl in 10 mM KH_2PO_4 buffer at pH 4.5 while the solution was magnetically stirred.

Fructose dehydrogenase is known to be highly specific for fructose,[5,23,24] and our Q-6/FDH electrode response was no different; no current was detected in the presence of glucose. Additionally, galactose, sucrose, lactose, maltose, xylose, arabinose, and sorbitol when added at 1 mM to a 1 mM solution of fructose did not affect the measured current. High sugar selectivity also was observed for an amperometric fructose sensor based on FDH immobilized on a carbon paste electrode containing benzoquinone; however, this system exhibited a strong, nonspecific response to ascorbic acid (see below).[6] Khan et al. have reported a nonspecific amperometric response to glucose and galactose on FDH−platinum and FDH−gold electrodes due to surface oxide formation in the presence of saccharides at the noble metal surface.[2,3] The addition of polypyrrole to their FDH−electrode construct eliminated this nonspecific response. Our negative results with glucose, galactose, and other sugars shows that the lipid/thiolate layer effectively blocks direct access of sugars to the gold electrode surface and that FDH is highly selective for fructose.

Prior to application of an FDH electrode to the measurement of fructose in citrus juice, the effects of electroactive ascorbic acid must be evaluated. Typically, ascorbic acid concentrations in citrus juice are 2−3% of the concentration of fructose.[5] Over a range of 0−1 mM ascorbic acid, a gold electrode modified with the membrane mimetic layer of chemisorbed thiols overlain with phospholipids showed ∼60-fold reduction in oxidative current response to ascorbic acid as compared to a bare gold electrode (see Figure 4). Figure 5 illustrates the effect of 0.1 mM ascorbic acid on the measured current of a 2 mM fructose solution. In this case, the presence of ascorbic acid at 5% of the fructose level resulted in an error of just 4%. In contrast, it has been reported that ascorbic acid at 2.5% of the fructose concentration results in a positive error of 80% for a sensor based on FDH immobilized on a carbon paste electrode.[6] The blocking lipid/thiolate layer of our system has the advantage of limiting access of ascorbic acid to the electrode surface. Figure 5 also shows the effect of ascorbic acid on the same electrode 22 days later. The resulting positive error had increased to 9%. This increase in error likely is due to an almost 40% drop in measured current for 2 mM fructose rather than degradation of the blocking lipid layer.

Figure 5 serves to illustrate the rapid response time of the Q-6/FDH electrode as well. Upon addition of 2 mM fructose to a stirred phosphate buffer solution, the steady-state current response generally was obtained within ∼20 s. Within 3 s, the electrode response is within 4% of the steady-state current. Within 11 s, the measured current is within 2% of steady state (1.2%). This performance is equivalent to or better than earlier FDH-based fructose biosensor prototypes.[2,3,5,6]

The stability of a Q-6/FDH electrode was evaluated over a period of six days. The electrode was used daily for many hours and was subjected to repeated rinsing. At the end of each day, the electrode was stored in buffer at 4 °C. The calibration curves for the first three days were virtually identical. Sensitivity decreased ∼10% on day four and remained unchanged on day five. On the sixth day, sensitivity dropped an additional 8%. This decline in sensitivity could be linked to loss of enzyme activity or leaching of coenzyme from the layer. However, earlier FDH immobilization results without phospholipid suggested that the loss of coenzyme is the primary factor, as re-introduction of coenzyme results in improved sensitivity.[16] Without the phospholipid layer, significant coenzyme was lost in hours, resulting in an unacceptable sensor lifetime of a day unless the quinone was replenished on the electrode by soaking in a buffer saturated with coenzyme Q. Results to date suggest that the useful lifetime of the electrode with the lipid layer is about two weeks, although enzyme activity has been evident after 35 days.

To test the electrode in a real sensing application, the fructose concentration in apple and orange juice was measured. A calibration curve was performed prior to each juice measurement. The juice samples were diluted in 10 mM KH_2PO_4, pH 4.5 buffer to fall in the linear range of the calibration curve. Results from three different electrodes were averaged and compared to those obtained with the enzymatic spectrophotometric assay kit. For apple juice, the electrodes yielded an average fructose concentration of 429 mM (n = 12, where n equals the number of juice measurements) compared to the assay kit value of 425 mM (n = 2, where n equals the number of assays). For orange juice, the electrodes measured an average fructose concentration of 127 mM (n = 3) compared to the enzyme assay kit value of 124 mM (n = 2). Relative standard deviations of 4.7 and 12.1% were obtained for the apple and orange juice samples, respectively. Although ascorbic acid levels were not determined independently for the

(24) Prado, F. E.; Sampietro, A. R. *Biotechnol. Appl. Biochem.* **1994**, *19*, 361−368.

juice samples, the close agreement between the electrode and assay kit measurements for fructose in orange juice offers encouraging evidence of ascorbic acid blocking in a real sample environment, as ascorbic acid typically is about 2−3% of the fructose concentration in citrus juice.[5]

An attempt was made to determine the enzyme coverage on the electrode surface. The plastic Kel-F material surrounding the gold disk was removed; the gold tip was placed in 0.1% Triton X-100 solution and was gently agitated to remove enzyme from the lipid/thiolate layer. The solution was then assayed, and the total enzyme activity was compared to that expected for monolayer coverage (as estimated by assuming spherical enzyme shape). For one electrode, an FDH coverage of 20% of a theoretical monolayer was estimated, which suggests that further substantial improvement in fructose biosensor activity and useful lifetime could be had through increased enzyme surface coverage.

CONCLUSION

An effective method of minimizing electroactive interference due to ascorbic acid in fructose measurements with a membrane mimetic Q-6/FDH electrode has been described. The membrane mimetic layer coating the gold electrode surface limits access of oxidizable ascorbic acid to the gold surface. It also serves as a medium for the stable immobilization of membrane-bound FDH. Ascorbic acid, at concentrations similar to those found in citrus juice, caused errors in fructose measurements of only a few percent. In fact, the average measurement of the fructose content of orange juice conducted with the biosensor was within 3% of the value determined with the enzyme assay kit. The prototype biosensor also exhibits rapid response time and good sensitivity. Although preliminary findings are encouraging, future improvements in stability and further study of its accuracy and precision may be necessary before this biosensor could be employed effectively for the repeated measurement of fructose in fruit juices.

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