Anal. Chem. **2004,** *76,* 78−85

A Glucose Biosensor Based on Surface-Enhanced Raman Scattering: Improved Partition Layer, Temporal Stability, Reversibility, and Resistance to Serum Protein Interference

Chanda Ranjit Yonzon,[†,‡] Christy L. Haynes,[†,‡] Xiaoyu Zhang,[†] Joseph T. Walsh, Jr.,[§] and Richard P. Van Duyne*[,†]

Department of Chemistry and Department of Biomedical Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113

This work updates the recent progress made toward fabricating a real-time, quantitative, and biocompatible glucose sensor based on surface-enhanced Raman scattering (SERS). The sensor design relies on an alkanethiolate tri(ethylene glycol) monolayer that acts as a partition layer, preconcentrating glucose near a SERS-active surface. Chemometric analysis of the captured SERS spectra demonstrates that glucose is quantitatively detected in the physiological concentration range (0−450 mg/dL, 0−25 mM). In fact, 94% of the predicted glucose concentrations fall within regions A and B of the Clarke error grid, making acceptable predictions in a clinically relevant range. The data presented herein also demonstrate that the glucose sensor provides stable SERS spectra for at least 3 days, making the SERS substrate a candidate for implantable sensing. Glucose sensor reversibility and reusability is evaluated as the sensor is alternately exposed to glucose and saline solutions; after each cycle, difference spectra reveal that the partitioning process is largely reversible. Finally, the SERS glucose sensor successfully partitions glucose even when challenged with bovine serum albumin, a serum protein mimic.

Improved glucose-sensing technology will yield a significant increase in the quality of life for the 17 million diabetics and 16 million prediabetics in the United States. Scientific advances will also considerably reduce the estimated $132 billion in annual economic cost of diabetes.[1] Diabetes mellitus is a disease characterized by elevated blood glucose levels caused by the body's abnormal response to or production of insulin, a hormone responsible for glucose metabolism regulation. The long-term health outlook of diabetic patients improves with frequent glucose measurements and careful control of glucose levels.

Numerous studies have been performed in recent years toward developing in vivo, minimally invasive, biologically compatible, and quantitative real-time glucose sensors.[2,3] Of these studies, electrochemical methods have been the most successful techniques, relying on the detection of hydrogen peroxide produced by the enzymatic oxidation of glucose.[4,5] One of the disadvantages of this indirect detection method is that glucose oxidase, the enzyme that catalyzes the oxidation, needs to be replenished and, therefore, limits the lifetime of the sensor. Another serious problem inherent to enzymatic glucose sensors is the lack of stability due to the intrinsic nature of enzymes.[6] To avoid the disadvantages of enzyme-based sensors, nonenzymatic detection of glucose has been recently developed using amperometric measurements on platinum electrodes.[6−9] However, this method has insufficient selectivity over interfering species in a biocompatible potential range.[6,9] Indirect detection of glucose is also done using fluorescence spectroscopy. The signal transduction mechanism in fluorescence sensors exploits the reversible glucose-binding characteristics of fluorophore-labeled concanavalin A (Con A); separation of the fluorophore from Con A yields a measurable signal.[10,11] Though this is a very promising technique that features a physiologically relevant detection range, biomolecules similar to glucose can interfere in this multistep process, giving false positives. Diffraction spectroscopy, another indirect detection method, has been implemented by Asher and co-workers to detect glucose in aqueous humor.[12,13] In this technique, the binding of glucose to boronic acid embedded in a polymerized crystalline

* To whom correspondence should be addressed: (e-mail) vanduyne@chem.northwestern.edu.

[†] Department of Chemistry.

[‡] These authors contributed equally to this work.

[§] Department of Biomedical Engineering.

(1) American Diabetes Association, http://www.diabetes.org/main/application/commercewf?origin=*.jsp&event=link(B1), access date 07−22−2003.

(2) McNichols, R. J.; Cote, G. L. *J. Biomed. Opt.* **2000**, *5*, 5−16.

(3) Steffes, P. G. *Diabetes Technol. Ther.* **1999**, *1*, 129−133.

(4) Tamada, J. A.; Garg, S.; Jovanovie, L.; Pitzer, K. R.; Fermi, S.; Potts, R. O. *J. Am. Med. Assoc.* **1999**, *282*, 1839−1844.

(5) Kaufman, F. R.; Gibson, L. C.; Halvorson, M.; Carpenter, S.; Fisher, L. K.; Pitukcheewanont, P. *Diabetes Care* **2001**, *24*, 2030−2034.

(6) Park, S. C., T. C.; Kim, H. C. *Anal. Chem.* **2003**, *75*, 3046−3049.

(7) Vassilyev, Y. B.; Khazova, O. A.; Nikolaeva, N. N. *J. Electroanal. Chem.* **1985**, *196*, 105−125.

(8) Beden, B.; Largeaud, F.; Kokoh, K. B.; Lamy, C. *Electrochim. Acta* **1996**, *41*, 701−709.

(9) Sun, Y. P.; Buck, H.; Mallouk, T. E. *Anal. Chem.* **2001**, *73*, 1599−1604.

(10) Russell, R. J.; Pishko, M. V.; Gefrides, C. C.; McShane, M. J.; Cote, G. L. *Anal. Chem.* **1999**, *71*, 3126−3132.

(11) Ballerstadt, R.; Schultz, J. S. *Anal. Chem.* **2000**, *72*, 4185−4192.

(12) Asher, S. A. A., V. L.; Goponenko, A. V.; Sharma, A. C.; Lednev, I. K.; Wilcox, C. S.; Finegold, D. N. *J. Am. Chem. Soc.* **2003**, *125*, 3332−3329.

10.1021/ac035134k CCC: $27.50
Published on Web 11/25/2003

colloidal array (PCCA) induces a change in osmotic pressure, swelling the PCCA and red-shifting the diffraction peak. However, glucose binds with boronic acid most efficiently when the pH is higher than 8.5, a nonphysiological condition.

The direct glucose detection methods developed so far include laser polarimetry[14] and vibrational spectroscopies.[15−19] The laser polarimetry detection method is based on the rotation of polarized light caused by glucose in aqueous humor. Unfortunately, other chiral constituents in this biological environment, such as ascorbate and albumin, interfere during measurements.[14] Further, the rotation caused by corneal birefringence, and eye movement-induced variation in this rotation, can greatly complicate the quantification of the glucose concentration in vivo.[2] Vibrational spectroscopic methods applied to date for glucose sensing include near-infrared absorption, normal Raman scattering (NRS), and surface-enhanced Raman scattering (SERS) spectroscopies. Both NRS and SERS yield unique vibrational signatures for small-molecule analytes, as well as quantitative information. The implementation of near- and mid-infrared spectroscopic methods has a fundamental limitation due to the competing absorption by water and spectral congestion. However, the application of multivariate calibration models presents a possible remedy.[17] Raman spectroscopy, in all its forms, is a vibrational spectroscopic method that has the inherent ability to distinguish between molecules with great structural similarity, molecules such as glucose and fructose.[20] Moreover, normal Raman spectroscopy has been shown to be able to detect physiological concentrations of glucose in vitro from a simulated aqueous humor solution.[18] However, high laser powers and long acquisition times are required due to the inherently small NRS cross section of glucose, 5.6×10^{-30} cm^2 molecule^{-1} sr^{-1}.[21] Higher intensity Raman signals and lower detection limits can be achieved using SERS. SERS produces very large enhancements in the effective Raman cross section of species spatially confined within range of the electromagnetic fields (viz. 0−4 nm)[22] generated upon excitation of the localized surface plasmon resonance of nanostructured noble metal surfaces. The Raman signals of ensemble-averaged molecules show enhancement of up to 8 orders of magnitude,[23] while the signals from single molecules can show an increase by 14 or 15 orders of magnitude in special cases.[24,25] In comparison with infrared and NRS spectroscopies, SERS enjoys both the advantage of application in aqueous media because of the small Raman scattering cross section of water[21] and the sensitivity for trace level detection.[26]

In our previous work,[19] the first systematic study of the direct detection of glucose was performed using SERS. Glucose was partitioned into a decanethiol monolayer adsorbed on a silver film over nanosphere (AgFON) surface and thereby preconcentrated within the zone of electromagnetic field enhancement. A chemometric method, specifically, leave-one-out partial least-squares (LOO-PLS) analysis, was used to demonstrate quantitative glucose detection in both large (0−4,500 mg/dL, 0−250 mM) and clinically relevant (0−450 mg/dL, 0−25 mM) concentration ranges. Chemometric methods, such as LOO-PLS, are exploited for analyte quantification when the spectrum of interest is embedded within a complex background spectrum.

The current work demonstrates effective glucose quantification with improved alkanethiolate selectivity for glucose over a blood serum protein mimic in aqueous humor by using (1-mercaptoundeca-11-yl)tri(ethylene glycol) (EG3) as the partition layer. In fact, the data presented herein demonstrate that the EG3-modified AgFON substrate provides stable SERS spectra for at least 3 days, making the SERS substrate a candidate for implantable sensing. The reusability of the SERS sensor is evident during experiments where the EG3-modified AgFON is alternately exposed to glucose and saline solutions. EG3 is known to form a 2-nm-thick monolayer on noble metal surfaces[27] and provides a resistance to adsorption of proteins and enzymes.[28−30] The ability of ethylene glycol-terminated polymers to reject nonspecific binding by background proteins[29−34] and its biocompatibility[35−37] have been at the center of interest in several studies. While the fundamental mechanism of such repulsion is still being debated, these highly valued properties are exploited here to fabricate an improved SERS glucose sensor. The experiments presented herein demonstrate that an EG3 partition layer has the capability of capturing glucose near the surface, while showing resistance to serum albumin, the most abundant protein in plasma.[38]

In our earlier publication introducing the possibility of a SERS-based glucose sensor,[19] we identified seven milestones to be achieved on the path to a fully functional, continuous, minimally invasive, quantitative, real-time, in vivo glucose sensor for application in biological media including aqueous humor, interstitial fluid, and blood. The first milestone, demonstration that quantitative

(13) Alexeev, V. L.; Sharma, A. C.; Goponenko, A. V.; Das, S.; Lednev, I. K.; Wilcox, C. S.; Finegold, D. N.; Asher, S. A. *Anal. Chem.* **2003**, *75*, 2316−2323.
(14) Cameron, B. D.; Gorde, H. W.; Satheesan, B.; Cote, G. L. *Diabetes Technol. Ther.* **1999**, *1*, 125−143.
(15) Klonoff, D. C.; Braig, J.; Sterling, B.; Kramer, C.; Goldberger, D.; Trebino, R. *IEEE LEOS Newsl.* **1998**, *12*, 13−14.
(16) Berger, A. J.; Koo, T. W.; Itzkan, I.; Horowitz, G.; Feld, M. S. *Appl. Opt.* **1999**, *38*, 2916−2926.
(17) Zhang, L.; Small, G. W.; Arnold, M. A. *Anal. Chem.* **2002**, *74*, 4097−4108.
(18) Lambert, J.; Storrie-Lombardi, M.; Borchert, M. *IEEE LEOS Newsl.* **1998**, *12*, 19−22.
(19) Shafer-Peltier, K. E.; Haynes, C. L.; Glucksberg, M. R.; Van Duyne, R. P. *J. Am. Chem. Soc.* **2003**, *125*, 588−593.
(20) Soderholm, S.; Roos, Y. H.; Meinander, N.; Hotokka, M. *J. Raman Spectrosc.* **1999**, *30*, 1009−1018.
(21) McCreery, R. L. *Raman Spectroscopy for Chemical Analysis*; John Wiley & Sons: New York, 2000.
(22) Schatz, G. C.; Van Duyne, R. P. In *Handbook of Vibrational Spectroscopy*; Chalmers, J. M., Griffiths, P. R., Eds.; Wiley: New York, 2002; Vol. 1, pp 759−774.
(23) Haynes, C. L.; Van Duyne, R. P. *J. Phys. Chem. B* **2003**, *107*, 7426−7433.
(24) Nie, S.; Emory, S. R. *Science* **1997**, *275*, 1102−1106.
(25) Kneipp, K.; Wang, Y.; Kneipp, H.; Perelman, L. T.; Itzkan, I.; Dasari, R. R.; Feld, M. S. *Phys. Rev. Lett.* **1997**, *78*, 1667−1670.
(26) Sylvia, J. M.; Janni, J. A.; Klein, J. D.; Spencer, K. M. *Anal. Chem.* **2000**, *72*, 5834−5840.
(27) Palegrosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 12−20.
(28) Prime, K. L.; Whitesides, G. M. *Science* **1991**, *252*, 1164−1167.
(29) Ostuni, E.; Chapman, R. G.; Liang, M. N.; Meluleni, G.; Pier, G.; Ingber, D. E.; Whitesides, G. M. *Langmuir* **2001**, *17*, 6336−6343.
(30) Lahiri, J.; Isaacs, L.; Tien, J.; Whitesides, G. M. *Anal. Chem.* **1999**, *71*, 777−790.
(31) Clark, S. L.; Hammond, P. T. *Adv. Mater.* **1998**, *10*, 1515−1519.
(32) Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 10714−10721.
(33) Zolk, M.; Eisert, F.; Pipper, J.; Herrwerth, S.; Eck, W.; Buck, M.; Grunze, M. *Langmuir* **2000**, *16*, 5849−5852.
(34) Dicke, C.; Hahner, G. *J. Am. Chem. Soc.* **2002**, *124*, 12619−12625.
(35) Mauzac, M. A., N.; Jozefonvicz, J. *Biomaterials* **1982**, *3*, 221−224.
(36) Lee, J. H.; Kopecek, J.; Andrade, J. D. *J. Biomed. Mater. Res.* **1989**, *23*, 351−368.
(37) Cohn, D. Y., H. *J. Biomed. Mater. Res.* **1988**, *22*, 993−1009.
(38) Baker, M. E. *FEBS Lett* **1998**, *439*, 9−12.

sensing of glucose using SERS could be done under any circumstances was accomplished therein. The work presented here demonstrates a set of four significant advances toward achieving these milestones: (1) replacement of the 1-decanthiol partition layer with an EG3 monolayer, (2) detection of glucose in the presence of serum albumin, (3) 3-day temporal stability of the EG3-modified AgFON surface, and (4) demonstration of the reversible nature of glucose partitioning. The remaining milestones yet to be achieved include the following: (1) glucose sensing in the presence of other small-molecule analytes, (2) fabrication of a AgFON substrate on the tip of a fiber-optic probe and in vivo testing of the SERS-active probe, (3) miniaturization of the implanted SERS-active surface, and (4) miniaturization of the SERS instrument. After achieving these milestones, one can envision the application of the SERS sensor as a biomedical glucose-sensing technology. Specifically, this miniaturized SERS substrate (microscale or nanoscale) will be implanted subcutaneously or incorporated as a component of a prosthetic lens in the eye, achieving glucose detection with external excitation and collection apparatus.

EXPERIMENTAL SECTION

Materials. All the chemicals were of reagent grade or better and used as purchased. Ag wire (99.99%, 0.04-in. diameter) was purchased from D. F. Goldsmith (Evanston, IL). Oxygen-free high-conductivity copper was obtained from McMaster-Carr (Chicago, IL) and cut into 18-mm-diameter disks. CH_3CH_2OH, H_2O_2, and NH_4OH were purchased from Fisher Scientific (Fairlawn, VA). Surfactant-free, white carboxyl-substituted latex polystyrene nanosphere suspensions (390 ± 19.5 nm diameter, 4% solid) were acquired from Duke Scientific Corp. (Palo Alto, CA). Tungsten vapor deposition boats were purchased from R. D. Mathis (Long Beach, CA). For substrate and solution preparations, ultrapure water (18.2 MΩ cm^{-1}) from a Millipore academic system (Marlborough, MA) was used. Bovine serum albumin (BSA), saline, and tris buffer (pH = 7.4) were obtained from Sigma (St. Louis, MO). The disposable filters with 0.45-μm pore size were acquired from Gelman Sciences (Ann Arbor, MI). (1-Mercaptoundeca-11-yl)tri(ethylene glycol) ($HS(CH_2)_{11}(OCH_2CH_2)_3OH$, EG3) was synthesized[27] and donated by the Mrksich group at the University of Chicago.[39]

AgFON Fabrication and Incubation Procedure. AgFON substrates were used because of their stable SERS activity in electrochemical,[40,41] ultrahigh vacuum,[42] and ambient experiments.[19] In this work, AgFONs were fabricated on copper substrates. The copper substrates were cleaned by sonicating in 10:1:1 H_2O/30% H_2O_2/NH_4OH. Approximately 12 μL of nanosphere solution was drop-coated onto a clean copper substrate and allowed to dry at room temperature. Then, 200-nm-thick Ag films were deposited onto and through the nanosphere mask using a modified Consolidated Vacuum Corp. vapor deposition system (base pressure 10^{-7} Torr).[43] The mass thickness and deposition rate (~1 nm/s) of the Ag metal were measured by a Leybold Inficon XTM/2 quartz-crystal microbalance (East Syracuse, NY). AgFON substrates were first incubated in 1 mM EG3 in ethanol for more than 12 h. Then, the EG3-modified substrates were mounted into a small-volume flow cell and exposed to glucose solutions for 10 min to ensure complete partitioning of the glucose into the EG3 monolayer.

Surface-Enhanced Raman Scattering Spectroscopy. A Spectra-Physics model 120 HeNe laser was used to produce the 632.8-nm excitation wavelength (λ_{ex}); the laser spot size was less than 2 mm in diameter. The SERS measurement system includes an interference filter (Edmund Scientific, Barrington, NJ), a holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI), a model VM-505 single-grating monochromator with the entrance slit set at 100 μm (Acton Research Corp., Acton, MA), and a LN_2-cooled CCD detector (Roper Scientific, Trenton, NJ). A collection lens with magnification 5 was used to collect the scattered light. The small-volume flow cell[44] was used to control the external environment of AgFON surfaces throughout the SERS experiment.

Chemometrics Method. All data processing was performed using MATLAB (MathWorks, Inc., Natick, MA) and PLS_Toolbox (Eigenvector Research, Inc., Manson, WA). Prior to analysis, cosmic rays were removed from the spectra using a derivative filter. The slowly varying background, commonly seen in SERS experiments, was removed mathematically by subtracting a fourth-order polynomial. This method minimally affected the SERS peaks while greatly reducing the background level. The SERS spectral intensities were normalized using the 1107-cm^{-1} peak from the EG3 partition layer spectrum. From each spectrum, the absolute peak intensity of the 1107-cm^{-1} peak was obtained by subtracting the peak from the background (at 1174 cm^{-1}). An average peak intensity was determined for each experiment. To obtain the normalization factor for each sample spectrum, the average peak intensity was divided by the absolute peak intensity measured in each spectrum. Each spectrum was normalized by multiplying the spectrum by the normalizing factor before chemometric analysis. Then, data analysis was performed using partial least-squares leave-one-out analysis.

UV−Visible Spectroscopy. Measurements were carried out in a model 5000 Cary UV−Vis−NIR spectrometer (Varian Inc., Palo Alto, CA) using deuterium lamps.

RESULTS AND DISCUSSION

Significant progress has been made toward achieving a real-time, minimally invasive, biocompatible SERS glucose sensor. In previous work by Van Duyne and co-workers,[19] decanethiol was used as a partition layer for glucose, but the required sensor characteristics of temporal stability, reversibility, and biocompatibility were not studied in detail. Herein, EG3 was chosen as a partition layer because of its biocompatibility and hydrophilic properties, progressing toward the long-term goal of fabricating an implantable glucose sensor. The EG3-modified AgFON substrate was exposed to various concentrations of glucose under physiological conditions, promoting preconcentration of glucose near the AgFON surface (Figure 1). After data analysis using LOO-

(39) Hodneland, C. D.; Lee, Y. S.; Min, D. H.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5048−5052.
(40) Dick, L. A.; McFarland, A. D.; Haynes, C. L.; Van Duyne, R. P. *J. Phys. Chem. B* **2002**, *106*, 853−860.
(41) Dick, L. A.; Haes, A. J.; Van Duyne, R. P. *J. Phys. Chem. B* **2000**, *104*, 11752−11762.
(42) Litorja, M.; Haynes, C. L.; Haes, A. J.; Jensen, T. R.; Van Duyne, R. P. *J. Phys. Chem. B* **2001**, *105*, 6907−6915.
(43) Hulteen, J. C.; Van Duyne, R. P. *J. Vac. Sci. Technol. A* **1995**, *13*, 1553−1558.
(44) Malinsky, M. D.; Kelly, K. L.; Schatz, G. C.; Van Duyne, R. P. *J. Am. Chem. Soc.* **2001**, *123*, 1471−1482.

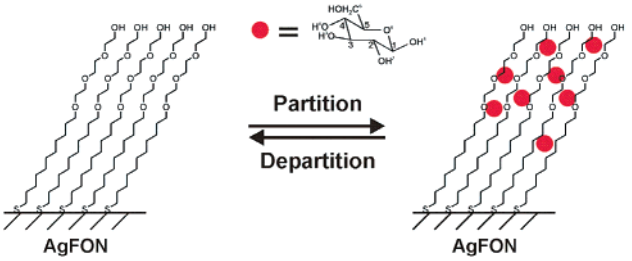

Figure 1. Schematic showing hypothetical glucose concentration gradient created by a (1-mercaptoundeca-11-yl)tri(ethylene glycol) (EG3) partition layer.

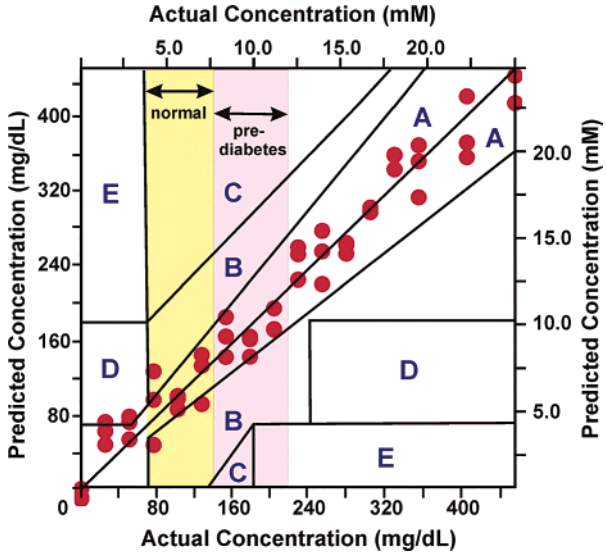

Figure 2. Clarke error grid of LOO-PLS predicted glucose concentration versus actual glucose concentration (5 loading vectors). AgFON samples were made (D = 390 nm, d_m = 200 nm), incubated for ~16 h in 1 mM EG3 solution, and dosed in glucose solution (range: 0−450 mg/dL, 0−25 mM) for 10 min. Each SERS measurement was made in the flow cell under saline with pH = 7.4, using λ_{ex} = 632.8 nm, P_{laser} = 2.5 mW, and t = 30 s.

PLS, the results are presented in a Clarke error grid (Figure 2). Clarke and co-workers established the Clarke error grid as the metric for evaluating glucose sensor efficacy in the clinical concentration range.[45] The Clarke error grid is divided into five major zones: zone A predictions lead to clinically correct treatment decisions; zone B predictions lead to benign errors or no treatment; zone C predictions lead to overcorrecting acceptable blood glucose concentrations; zone D predictions lead to dangerous failure to detect and treat; and zone E predictions lead to further aggravating abnormal glucose levels.

Quantitative Study of Glucose Using EG3 Partition Layer. A viable glucose biosensor must be capable of detecting 0−450 mg/dL (0−25 mM) glucose under physiological conditions. Toward this goal, each EG3-modified AgFON sample was incubated for 10 min in a pH = 7.4 saline solution containing glucose concentrations from 0 to 450 mg/dL (0−25 mM). The samples were placed in an environmental control flow cell under saline, and SERS spectra were then measured (λ_{ex}= 632.8 nm, P_{laser} = 2.5 mW, t = 30 s). After spectral normalization using EG3 peak intensities, the SERS spectra were analyzed with the LOO-PLS

(45) Clarke, W. L.; Cox, D.; Gonder-Frederick, L. A.; Carter, W.; Pohl, S. L. *Diabetes Care* **1987**, *10*, 622−628.

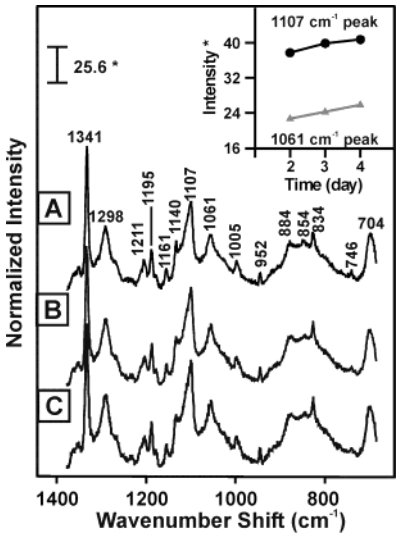

Figure 3. SERS spectra demonstrating the stability of an EG3-modified AgFON surface for at least 3 days. (A) After ~24 h of incubation in saline with pH = 7.4, λ_{ex} = 632.8 nm, P_{laser} = 1.3 mW, and t = 60 s. (B) After ~48 h of incubation in saline with pH = 7.4, λ_{ex} = 632.8 nm, P_{laser} = 1.3 mW, and t = 60 s. (C) After ~72 h of incubation in saline with pH = 7.4, λ_{ex} = 632.8 nm, P_{laser} = 1.2 mW, and t = 60 s. Inset shows the intensity variation for 1107- and 1061-cm^{-1} peaks with time. * denotes adu mW^{-1} s^{-1}.

method. In the data presented in Figure 2, five loading vectors were found to minimize the root-mean-squared error of cross-validation (RMSECV). The resulting cross-validated glucose concentration predictions are presented in the Clarke error grid (Figure 2).

The EG3-modified AgFON sensor quantitatively detects glucose in the physiological range with a corresponding RMSECV of 82 mg/dL (4.5 mM). In Figure 2, 94% of the predictions fall in zones A and B, while a few data points overlap in zone D within the hypoglycemic area (<70 mg/dL, <3.9 mM). The cross-validation error of 82 mg/dL (4.5 mM) can be partially attributed to variation of the SERS enhancement factor on different AgFON samples. The nanostructure on a AgFON substrate varies from point to point, affecting the localized surface plasmon resonance and, accordingly, the SERS enhancement factor.[23] In an effort to reduce the cross-validation error, the data later presented in Figure 7 used a single AgFON sample with a single point of detection. In addition to demonstrating quantitative glucose detection in a clinically relevant concentration range, other characteristics of the EG3-modified AgFON glucose sensor, such as durability, reusability, and selectivity, also need to be evaluated.

Temporal Stability of the EG3-Modified Substrate. Implantable glucose sensors must be stable for at least a 3-day period.[5] Previous work has demonstrated that bare AgFON surfaces display extremely stable SERS activity when challenged with high potentials[40] and high temperatures in ultrahigh vacuum.[42] Here, the stability of the EG3-modified AgFON SERS substrate is studied over a period of 3 days in saline with pH = 7.4 at room temperature. SERS spectra were captured every 24 h from the same sample location (λ_{ex} = 632.8 nm, t = 60 s) (Figure 3). The EG3 spectral band positions do not vary significantly over the course of 72 h. However, peaks at 1107 and 1064 cm^{-1} increase in intensity by 7.5 and 13% over 48 h, respectively (inset in Figure 3). The molecular order of self-assembled monolayers (SAMs)

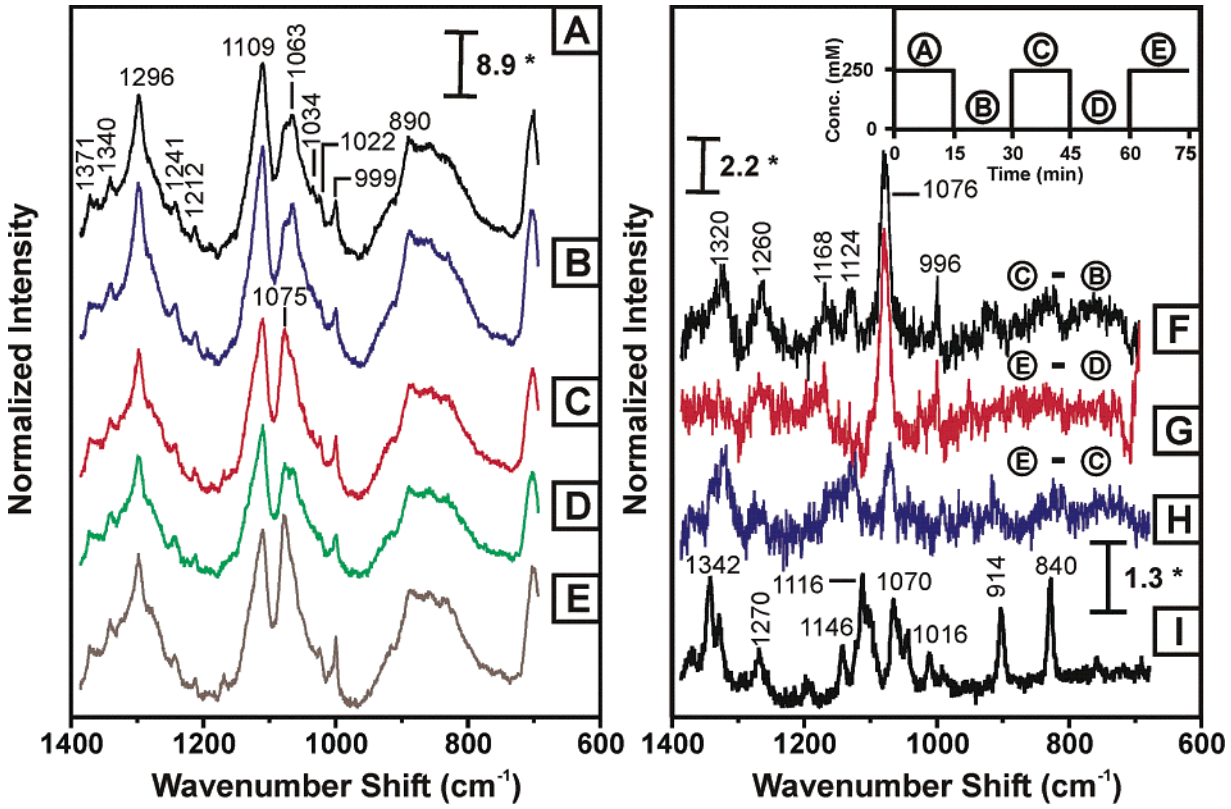

Figure 4. SERS spectra demonstrating the partition/departition capability of the EG3-modified AgFON substrate. The left panel displays SERS spectra of the sample cycled between 250 and 0 mM glucose solutions using $\lambda_{ex} = 632.8$ nm, $P_{laser} = 1.5$ mW, and $t = 30 \times 20$ s. (F) and (G) Difference spectra illustrating the glucose partitioning obtained by subtracting (B) from (A) and (D) from (E), respectively. (H) Difference spectra illustrating the glucose departitioning obtained by subtracting (C) from (E). Inset shows the schematic of the partition/departition concentration pulse experiment. (I) NRS of crystalline glucose for comparison, $\lambda_{ex} = 632.8$ nm, $P_{laser} = 5$ mW, and $t = 30$ s. * denotes adu mW^{-1} s^{-1}.

increases with incubation time;[46] the rearrangement of the SAM gives rise to peaks with increasing intensity. The SERS peaks at 1341 and 834 cm^{-1} have been identified as a signature of highly ordered SAMs[47,48] and are the subject of further investigation.

Reversible Glucose Sensing. While the quantitative detection of glucose using the EG3-modified AgFON sensor and the stability of the sensor has been demonstrated, an implantable sensor must also be reusable. To examine the partition/departition capability of the EG3-modified AgFON sensor, it was exposed to cycles of 250 and 0 mM glucose solutions without flushing the sensor with saline between measurements (Figure 4 inset). SERS spectra were captured after each concentration variation ($\lambda_{ex} = 632.8$ nm, $P_{laser} = 1.5$ mW, $t = 30 \times 20$ s) (Figure 4, left panel). Traces F and G of Figure 4 are the difference spectra representing glucose partitioned into the EG3 SAM. Figure 4I is the Raman spectrum of crystalline glucose for comparison. Vibrational modes at 1342 (C−C−H bend), 1270, 1164, 1116 (C−C + C−O stretch), 1070 (C1−OH stretch), 914 (O−C1−H1 bend), and 840 cm^{-1} (C−C stretch) are known to be signatures of crystalline glucose.[20] The literature has shown that SERS spectral bands shift up to 25 cm^{-1} when compared to the NRS bands of the same analyte.[49] Peaks in the SERS difference spectrum (Figure 4F) at 1320, 1260, 1168, 1124, and 1076 cm^{-1} correspond with the Raman spectrum of crystalline glucose. To evaluate the glucose departitioning, spectral subtraction of two glucose-containing cycles was performed (Figure 4H). Figure 4H shows spectral features that match with the glucose peaks at 1320 and 1076 cm^{-1}, but with lower intensities. Based on the 1076-cm^{-1} peak area, up to 33% of the glucose may remain in the EG3 layer after the 0 mM glucose cycle. The high glucose concentration used in this experiment caused incomplete departitioning after each cycle, and accordingly, the glucose accumulated in each step. However, physiological concentrations (0−450 mg/dL, 0−25 mM) of glucose will not likely cause such accumulation in the partition layer, and the natural flow of aqueous humor[50] or interstitial fluid will assist glucose departitioning.

Selectivity of the Sensor for Glucose in the Presence of Blood Serum Protein Mimic. Quantitative detection, temporal stability, and reusability are important characteristics of a viable biosensor; however, the glucose sensor must be effective in the presence of interfering proteins. Serum albumin is a blood serum protein mimic for challenging the glucose sensor. Prior to use, BSA solutions were filtered using track-etch membranes with 0.45-μm-diameter pores to remove any undissolved particulate, and UV absorption spectra were taken to determine the BSA concentration. BSA solutions of 0.00, 0.63, 1.25, and 2.50 mg/mL were made

(46) Biebuyck, H. A.; Bain, C. D.; Whitesides, G. M. *Langmuir* **1994**, *10*, 1825−1831.
(47) Clark, B. K.; Gregory, B. W.; Avila, A. *J. Phys. Chem. B* **1999**, *103*, 8201−8204.
(48) Gregory, B. W.; Clark, B. K.; Standard, J. M.; Avila, A. *J. Phys. Chem. B* **2001**, *105*, 4684−4689.
(49) Stacy, A. M.; Van Duyne, R. P. *Chem. Phys. Lett.* **1983**, *102*, 365−370.
(50) Vanlandingham, B. D.; Brubaker, R. F. *Am. J. Ophthalmol.* **1998**, *126*, 191−196.

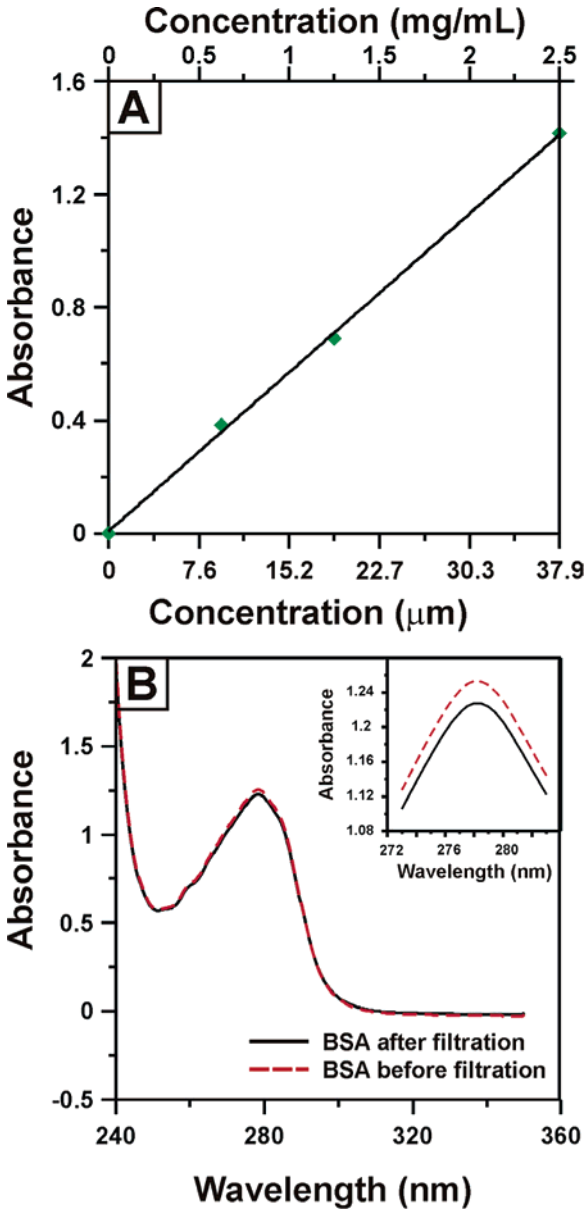

Figure 5. BSA concentration determination using UV absorption. (A) Calibration using 0.00, 0.63,1.25, and 2.50 mg/mL BSA in saline, pH = 7.4. (B) UV absorption of 33.2 μM (2.19 mg/mL) BSA before and after filtration using track-etch membranes with 0.45-μm-diameter pores. The inset magnifies the absorption difference between the filtered BSA and nonfiltered BSA.

to create a Beer−Lambert calibration curve with R^2 of 0.9990 (Figure 5A). The molar extinction coefficient at 277 nm, ϵ_{277} of BSA was calculated to be 3.7×10^4 M^{-1} cm^{-1}. This correlates with literature values ranging from 4.36×10^4 to 2.77×10^4 at wavelengths 279−288 nm.[51] Figure 5B shows the UV absorption spectra of 33.2 μM (2.19 mg/mL) BSA before and after filtration. The calculated BSA concentration after filtration was 32.6 μM (2.15 mg/mL), indicating a loss of 1.8% during filtration through 0.45-μm-diameter pores. The inset in Figure 5B magnifies the absorption difference between the BSA before and after filtration.

Figure 6A shows the SERS spectrum of the EG3-modified AgFON substrate in saline (λ_{ex} = 632.8 nm, P_{laser} = 0.8 mW, t = 240 s). When the serum albumin solution was injected into the flow cell, the SERS spectrum was collected throughout the 240-s incubation (Figure 6B). Finally, the sample was exposed to 100 mM glucose, and the SERS spectrum was collected (Figure 6C). Figure 6D is the difference spectrum demonstrating that serum albumin does not have a measurable SERS spectrum. The lack of SERS serum albumin bands could be due either to the small Raman scattering cross section of serum albumin or to the inefficient adsorption of serum albumin to the EG3 partition layer. Figure 6E demonstrates that the SERS glucose sensor is still effective after substrate exposure to an interfering protein. The peaks at 1449, 1433, 1339, 1291, 1108, 1077, 1059, and 855 cm^{-1} (Figure 6E) correspond with the crystalline glucose peaks shown in Figure 6F. Note that the glucose difference spectrum in Figure 6E has an altered appearance when compared to the glucose difference spectra shown in Figure 4F and G. This is the case because difference spectra highlight the glucose features versus the background spectral features. Varied background solution components yield varied glucose difference spectra. As long as the spectral locations of the peaks are comparable to the bulk glucose spectrum, it is fair to conclude that glucose is present. This experiment shows that glucose partitioning into EG3 is not affected by the presence of large molecules such as serum albumin. The peak at 695 cm^{-1} (Figure 6A) shifts to 710 cm^{-1} (Figure 6C) in the presence of glucose. This shift may be due to the rearrangement of the SAM when the glucose molecules partition into EG3. The observed shift further supports the hypothesis of glucose penetrating deeply into the EG3 monolayer, affecting even the character of the C−S bond.

Further studies of the effects of interfering protein were performed using glucose solutions (25−450 mg/dL, 1.4−25 mM) made in pH = 7.4 tris buffer containing 2 mg/mL serum albumin (filtered before addition). The EG3-modified AgFON was placed in a flow cell with the glucose solution, and SERS spectra were then measured (λ_{ex}= 632.8 nm, P_{laser} = 3 mW, t = 60 s). After spectral capture, the glucose solution was flushed from the flow cell, and the next glucose solution was injected. The next spectrum was captured from the same spatial location on the AgFON after 10-min incubation. The AgFON sensor was not flushed with saline between incremental glucose measurements in order to mimic the characteristics of in vivo measurements. After spectral normalization using EG3 peak intensities, the SERS spectra were analyzed with the LOO-PLS method. In the data presented in Figure 7, four loading vectors were found to minimize the RMSECV. The resulting cross-validated glucose concentrations are presented in the Clarke error grid format (Figure 7).

This work demonstrates that the EG3-modified AgFON sensor can be used to quantitatively detect glucose in the presence of serum albumin in the physiological range with a RMSECV of 54 mg/dL (3 mM). In Figure 7, nearly all the predictions lie in zones A and B, with the exception of one data point, which lies in zone D within the hypoglycemic region of the Clarke error grid (<70 mg/dL, < 3.9 mM). When comparing the RMSECV calculated in Figure 7 to that calculated in Figure 1, great improvement is apparent when a single sample and a single detection point are used. The RMSECV in Figure 7 may be further improved by eliminating excitation laser power fluctuation and solution turbulence present in the cell. In addition, the longevity of the EG3-

(51) Fasman, G. D. *Handbook of Biochemistry and Molecular Biology*, 3rd ed.; CRC Press Inc.: Cleveland, OH, 1976.

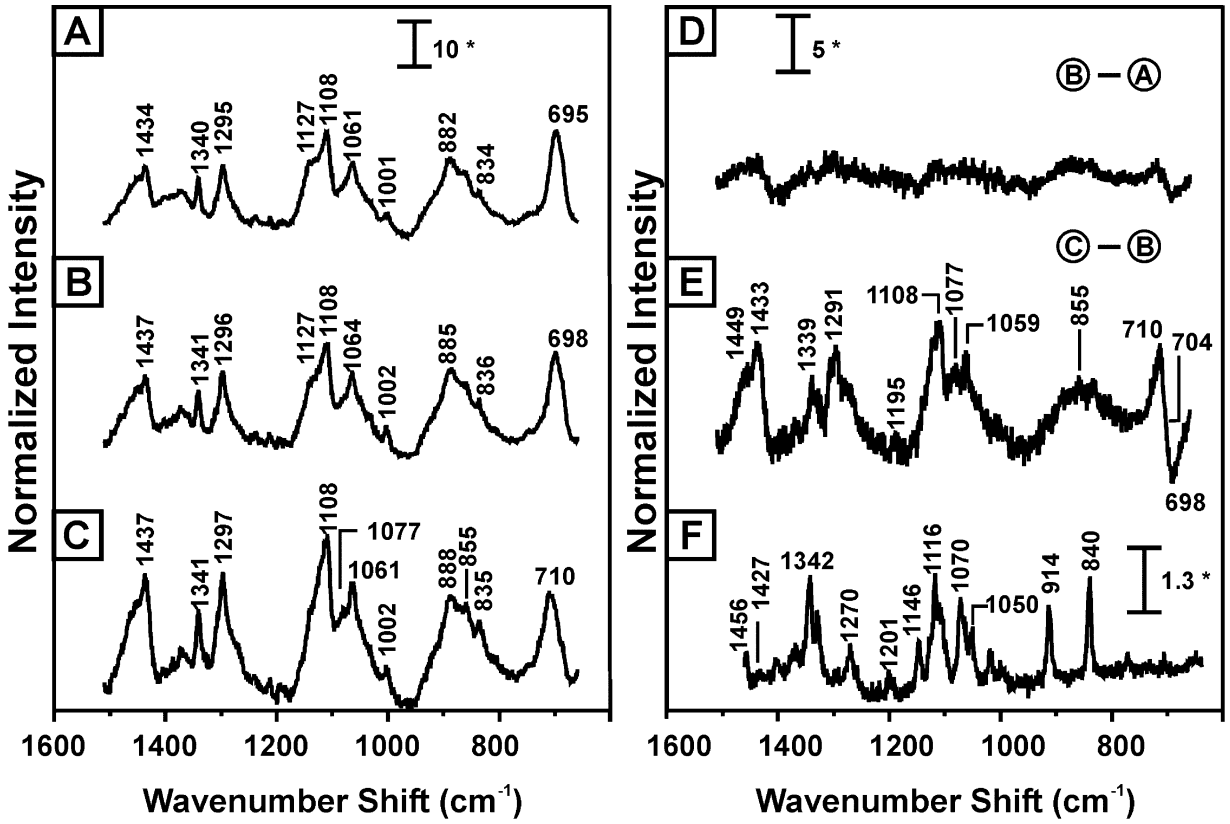

Figure 6. SERS spectra showing detection of glucose in the presence of serum albumin. (A) EG3 monolayer on AgFON substrate, λ_{ex} = 632.8 nm, P_{laser} = 0.8 mW, and t = 240 s. (B) A 1 mg/mL solution of serum albumin injected into the flow cell to challenge the EG3-modified AgFON, λ_{ex} = 632.8 nm, P_{laser} = 0.8 mW, and t = 240 s. (C) A 100 mM solution of glucose injected into the flow cell, λ_{ex} = 632.8 nm, P_{laser} = 0.8 mW, and t = 240 s. (D) Difference spectrum obtained by subtracting (A) from (B) revealing the lack of SERS spectrum for adsorbed serum albumin. (E) Difference spectrum obtained by subtracting (B) from (C) indicating serum albumin exposure does not interfere with glucose detection. (F) Normal Raman spectrum of crystalline glucose for comparison, λ_{ex} = 632.8 nm, P_{laser} = 5 mW, and t = 30 s. * denotes adu mW^{-1} s^{-1}.

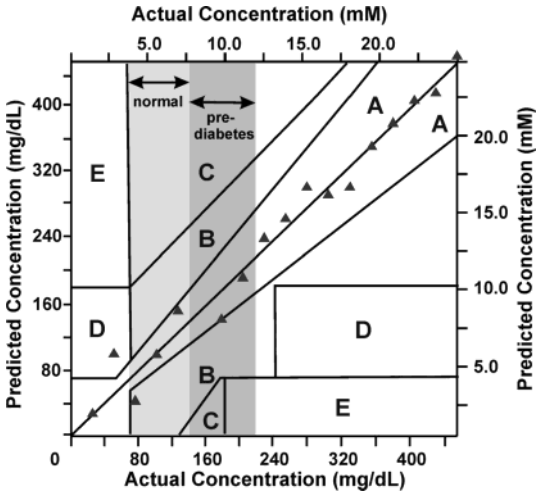

Figure 7. Clarke error grid of LOO-PLS predicted glucose concentration versus actual glucose concentration in the presence of 2 mg/mL BSA (4 loading vectors). An AgFON sample was made (D = 390 nm, d_m = 200 nm), incubated in 1 mM EG3 solution for ~16 h, and dosed in glucose solution (range: 25−450 mg/dL, 1.4−25 mM) with 2 mg/mL BSA for 10 min. Each SERS measurement was made in the flow cell under tris buffer with pH = 7.4, using λ_{ex} = 632.8 nm, P_{laser} = 3 mW, and t = 60 s.

modified AgFON surface was investigated by incubating the substrate in 2 mg/mL BSA for 6 h during data collection. Measured SERS spectra indicate that the surface is durable as well as SERS-active even when exposed to the blood serum protein mimic for several hours. Future studies will investigate 3-day exposure to the blood serum protein mimic before detection of glucose.

CONCLUSIONS

Significant progress has been made toward achieving a real-time, quantitative, and biocompatible SERS glucose sensor. In our previous work, decanethiol was used as an effective partition layer for glucose, but the required sensor characteristics of stability, reversibility, and selectivity were not studied in detail. This technology is further advanced by demonstration of quantitative detection of glucose in the physiological range (0−450 mg/dL, 0−25 mM) under physiological conditions, 3-day sensor stability, partition/departition efficacy of the sensor using an optimized partition layer, and glucose detection in the presence of an interfering protein.

The accuracy of the SERS glucose sensor is evaluated using the Clarke error grid, the accepted metric for judging the prediction capability of glucose sensors in the clinical concentration range.[45] In fact, 94% of the predictions fall in zones A and B, signifying that correct treatment choices can be made using this sensor. Additionally, the EG3-modified AgFON sensor quantitatively detects glucose in the physiological range with a corresponding cross-validation error of 82 mg/dL (4.5 mM). A large

portion of the calculated error can be attributed to the varying SERS enhancement factors due to sample-to-sample variation.

The stability of the EG3-modified AgFON SERS substrate is evident as the SERS bands and intensities do not change significantly during a 3-day incubation period in saline with pH = 7.4 at room temperature. The molecular order of the EG3 SAM increases with incubation time,[46] and this rearrangement gives rise to slightly larger SERS intensities.

Moreover, the glucose partition/departition capability of the EG3-modified AgFON sensor is demonstrated by exposing the sensor to cycles of 250 and 0 mM glucose solutions. The relatively high glucose concentration used in this experiment caused incomplete departitioning after each cycle, and accordingly, the glucose accumulated in each step. However, physiological concentrations of glucose will not likely cause such accumulation in the partition layer, and the natural flow of aqueous humor[50] or interstitial fluid will assist glucose departitioning. In future experiments, simulation of aqueous humor flow and more advanced difference spectroscopy methods will be applied.

Finally, this work also demonstrates that an EG3 partition layer can preconcentrate glucose near the surface quantitatively, while showing resistance to serum albumin, the most abundant protein in plasma.[38] In future work, glucose sensing will be performed in the presence of other interfering proteins and analyte mixtures found in aqueous humor, interstitial fluid, and blood.

ACKNOWLEDGMENT

The authors acknowledge the technical assistance of Adam D. McFarland, Dr. Douglas Stuart, and Ms. Olga Lyandres. The authors are grateful to Professor Milan Mrksich and Dr. Eunhee Jeoung at the University of Chicago for the donation of the EG3 and to Professor Joeseph T. Hupp at Northwestern University for access to the Cary UV-visible spectrometer. This project was supported by the Institute for Bioengineering and Nanoscience in Advanced Medicine at Northwestern University, the National Institutes of Health (EY13002 and EY13015), the National Science Foundation (EEC-0118025 and DMR-0076097), the Air Force Office of Scientific Research MURI program (F49620-02-1-0381), and a Northwestern University Presidential Fellowship (C.L.H.).

Received for review September 26, 2003. Accepted October 24, 2003.

AC035134K