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In Vivo Glucose Measurement by Surface-Enhanced Raman Spectroscopy

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This paper presents the first in vivo application of surface-enhanced Raman scattering (SERS). SERS was used to obtain quantitative in vivo glucose measurements from an animal model. Silver film over nanosphere surfaces were functionalized with a two-component self-assembled monolayer, and subcutaneously implanted in a Sprague−Dawley rat such that the glucose concentration of the interstitial fluid could be measured by spectroscopically addressing the sensor through an optical window. The sensor had relatively low error (RMSEC = 7.46 mg/dL (0.41 mM) and RMSEP = 53.42 mg/dL (2.97 mM).

Since the discovery of surface-enhanced Raman scattering in the 1970s, the number of publications on surface-enhanced Raman spectroscopy (SERS) has grown almost exponentially. Whereas the number of applications employing normal Raman scattering have dramatically increased, those using SERS have languished. SERS has been largely confined to fundamental or rarified academic research or denigrated as a "solution in search of a problem". For example, while it would seem that SERS would be ideally suited to the in situ study of catalytic reactions, where detailed vibrational information about transitional species would be highly advantageous, SERS has failed to materialize as a widespread analytical technique. Similarly, it has been difficult to effectively use SERS in bioanalysis. While indirect application of SERS, i.e., as a detection modality for molecular probes or labels, has been successful, attempts to use SERS to detect native biomolecules in situ has been limited.[1−3] Detection of nonresonant molecules of biological relevance, such as glucose, is challenging. There are pervasive hurdles to the more extensive use of SERS detection: (1) the historic view that both SERS signals and SERS-active surfaces exhibit poor reproducibility, (2) not all molecules are highly SERS active, (3) analytes must be close to (∼1−2 nm) or adsorbed on a roughened metal surface, and (4) the complexity and structural similarity of many important molecules (e.g., proteins) yield SERS spectra that are difficult to interpret.[4−6]

The challenge is even greater when attempting to obtain relevant SERS data in living systems. A nontrivial problem is placement of the SERS-active surface in vivo without damage to either the host or the surface. For example, colloids are difficult to introduce into cells and can aggregate in extracellular space. Solid substrates are more robust but require surgical implantation. Additional problems can manifest after a surface is surgically implanted and immersed in a biological milieu. There is currently no control over what species adsorb, perhaps irreversibly, to the SERS-active surface. This condition creates undesired spectral noise from nontarget molecules, while simultaneously blocking the access of the desired species, thereby lowering the analyte's overall signal. The cellular and in vivo environments are awash with a multitude of unknown interferants whose presence and concentration are in a constant state of flux. Indeed, the concentration of the target molecule itself is invariably changing. In an in vivo animal model, the problems are compounded by the host's immune response, the clotting factors, and the low concentration of target species in the extracellular matrix. These factors can contribute to surface contamination and cause unwanted effects.

We have developed a technique that addresses the critical problems previously limiting the use of SERS to glucose measurements, particularly in vivo. The fundamental enabling advances are the development of stable and strongly enhancing SERS-active surfaces and the chemical functionalization of those surfaces with self-assembled monolayers (SAMs).[7−10] The SAMs perform multiple roles in the detection system: limit fouling,[11,12] provide a convenient internal standard,[13] exclude entire classes of interferants,[14] and improve the glucose signal.[7−9,15−17]

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Stability of the SERS signal and surface stability are primarily determined by the material properties of the enhancing surface.[17−20] We have theoretically predicted and experimentally verified the parameters required to optimize the plasmonic properties of the film over nanospheres (FON)-type surfaces. Although the FON variant of nanosphere lithography (NSL) is intrinsically less enhancing (EF = 10^6) than other NSL varieties (10^8), FONs provide higher overall SERS signals.[20] This is because the total signal is related to both the SERS EF and the number of analyte molecules probed, which is quite high for FONs because of their relatively large viable surface area. The high radius of curvature imparted by the underlying nanospheres prevents annealing or loss of the nanoscale roughness features that give rise to SERS. The use of SAM-functionalized surfaces allows us to surmount the other hurdles.[9,21]

The SAM improves the signal from the analyte by partitioning glucose and localizing it within the first few nanometers of the SERS-active Ag or Au surface. This is critical, since SERS has a short-range distance dependence and can thus only detect analytes within this narrow zone.[22,23] Previously, we demonstrated the efficacy of an ω-alkanethiol (i.e., decanethiol (DT)) and a glycolated alkanethiol (1-mercaptoundeca-11-yl triethylene glycol (EG3)), as a partitioning SAM in the SERS measurement of glucose.[8,15,16] An important improvement is the development of a new mixed SAM based on two commercially available components, DT and mercaptohexanol (MH). The DT/MH SAM was designed to have dual hydrophobic/hydrophilic functionality, analogous to the ethylene glycol-terminated SAMs used in our previous work. In a recent publication, we conclusively demonstrate that the DT/MH mixed SAM performs better as a partitioning layer than the DT and EG3 SAMs used previously.[9] We conjecture, based on space-filling models, that the shorter hydroxyl-terminated chains form hydrophilic pockets, thus partitioning glucose closer to the SERS-active surface than the previously used SAMs. The SAM also excludes nontarget molecules, such as proteins, that could give rise to spectral congestion. This facilitates detection by simplifying the composition of the solution at the surface, while the SERS spectra provide a vibrational "fingerprint" that is unique to each molecule. It is this best-to-date partition layer that was used in the in vivo experiments.

EXPERIMENTAL SECTION

Materials. All the chemicals were reagent grade or better and used as purchased. Silver pellets (99.99%) were purchased from Kurt J. Lesker Co. (Clairton, PA). Oxygen-free, high-conductivity copper was obtained from McMaster-Carr (Chicago, IL) and cut into 18-mm-diameter disks. An 18-mm-diameter Cu mesh was also used effectively as a substrate. To clean substrates, NH_4OH, H_2O_2, and ethanol from Fisher Scientific (Fairlawn, VA) were used. Surfactant-free, white carboxyl-substituted latex polystyrene nanosphere suspensions (390 ± 19.5-nm diameter, 4% solid) were purchased from Duke Scientific Corp. (Palo Alto, CA). Ultrapure water (18.2 MΩ cm^{-1}) from a Millipore system (Marlborough, MA) was used for surface, substrate, and solution preparation. Glucose was purchased from Sigma (St. Louis, MO). Decanethiol ($CH_3(CH_2)_9SH$), and 6-mercapto-1-hexanol ($HS(CH_2)_6OH$) were purchased from Aldrich (Milwaukee, WI).

AgFON Fabrication and Incubation Procedure. The SAM-functionalized SERS-active surfaces were prepared in four steps, as shown in Figure 1B. First, silver film over nanosphere (AgFON) surfaces were fabricated by drop-coating 10 μL of 390-nm-diameter nanosphere solution onto clean copper substrates and then depositing a 200-nm-thick Ag film onto the nanosphere mask. This thickness provided the optimal LSPR location (Figure 1D) for SERS enhancement with 785-nm excitation.[20] The AgFON surfaces were then incubated in 1 mM DT for 45 min and subsequently transferred to 1 mM MH solution for at least 12 h to form a mixed DT/MH SAM. This produces a mixed monolayer in which DT predominates. The FONs were kept in 1 mM MH in ethanol prior to surgically implantation.

LSPR Reflectance Spectroscopy. Measurements were carried out using a SD2000 spectrometer coupled to a reflection probe (Ocean Optics, Dunedin, FL) and a halogen lamp (F-OLiteH, World Precision Instruments, Sarasota, FL). The reflection probe consists of a tight bundle of 13 optical fibers (12 illumination fibers around a collection fiber) with a usable wavelength range of 400−900 nm. All reflectance spectra were collected against a mirror-like Ag film over glass surface as a reference.

Surgical Implantation. The surgical procedure followed the protocol filed with Northwestern University and IACUC. Sprague−Dawley rats (300−500 g, $N = 4$) were anesthetized with pentobarbital (Office of Research Safety, Northwestern University) with an initial dose of 50 mg/kg. The animals were checked for pain reactions by toe tug and blink tests. The rats were kept under anesthetic by hourly administration of pentobarbital at 25 mg/kg. After the anesthetic had taken effect, the surgical areas were prepared by removal of hair (shaving and chemical depilatory) and cleaning. Then, the femoral vein was cannulated using PE 50 tubing (Clay Adams) for glucose injections. The carotid artery was cannulated with PE 90 tubing for blood glucose measurements with FDA-qualified home medical equipment (One Touch II Meter, Lifescan, Inc.). A tracheotomy was performed to enable the attachment of a ventilator to aid respiration. The incisions were shut with surgical clips. The rat was thermally stabilized by an electric heating pad throughout the course of the surgery and experiment. A metal frame containing a glass window was placed along the midline of the rat's back. A circular incision was made to allow the positioning of a DT/MH-functionalized AgFON sensor subcutaneously such that the substrate was in contact with the interstitial fluid and optically addressable through the window. The rodent was then gently positioned into a heated holder in the conventional sample position on a lab-scale Raman spec-

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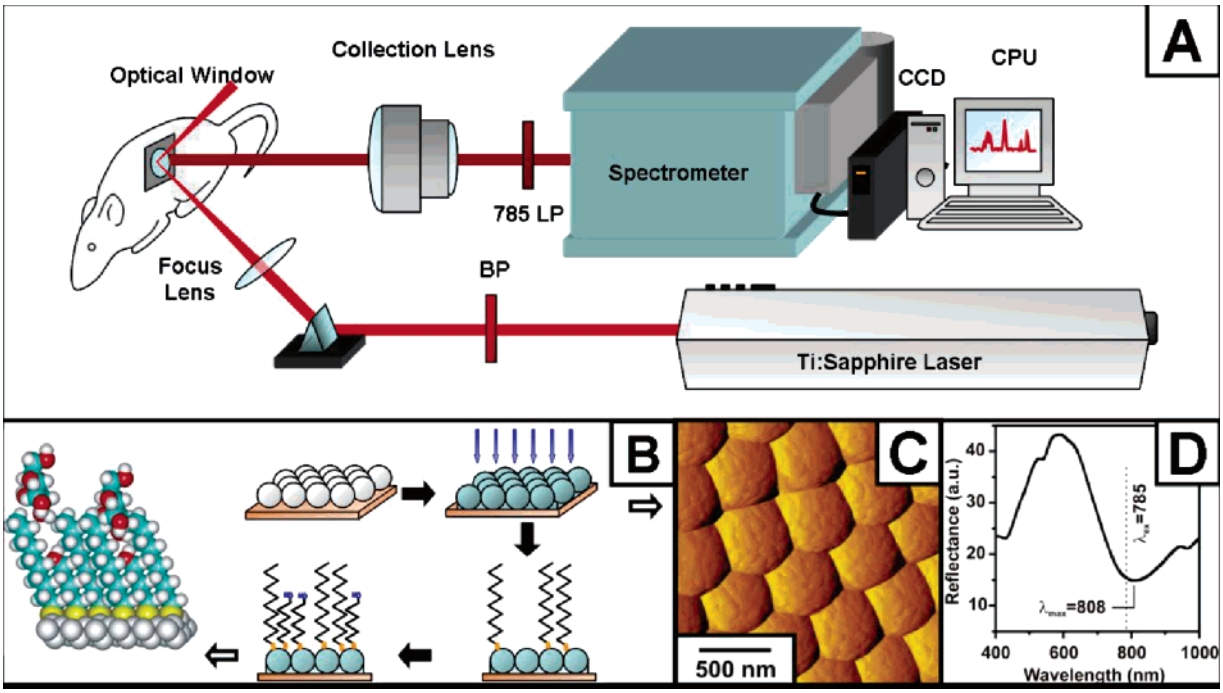

Figure 1. Schematic of (A) instrumental apparatus, (B) sensor preparation, (C) morphology, and (D) optical characterization. (A) A rat with a surgically implanted sensor and optical window was integrated into a conventional laboratory Raman spectroscopy system consisting of a Ti:sapphire laser (λ = 785), band-pass filter, steering and collection optics, and a long-pass filter that rejected Raleigh scattered light. (B) AgFONs were prepared by depositing metal through a mask of self-assembled nanospheres. The AgFON was then functionalized by successive emersions in ethanolic solutions of decanethiol and mecaptohexanol. Glucose is able to partition in to and out of the DT/MH layer, as shown in the left of the frame (C) The resultant structure is show in the atomic force micrograph. (D) After functionalization, a reflectance spectrum was collected to determine the position of the LSPR.

troscopy system (Figure 1A). This system consists of a laser as a monochromatic light source, a band-pass filter to clean up the laser line, a series of steering, focusing optics to deliver the laser light to the sample, and collection optics, which relay the scattered light to the detector, a high-quality, long-pass filter to reject Rayleigh scattering, a third meter spectrograph for wavelength dispersion, and a CCD detector. Following the experiment, the animals were sacrificed with an overdose of anesthetic and bilateral thorachotomy, according to protocol.

Experimental Procedure and Spectroscopic Measurement. Glucose was varied in the rat through intermittent intravenous infusion for 3 h. An infusion of glucose was delivered over 5−10 min, at a concentration of 1 g/mL in sterile phosphate-buffered saline via the femoral cannula. A droplet of blood was drawn from the rat, the glucose level was measured with the One Touch II glucometer, and corresponding SERS measurements were taken. The SERS spectra were acquired through the optical window using a Ti:sapphire laser (λ_{ex} = 785 nm, P = 50 mW, t = 2 min). The data were collected and analyzed by the partial least-squares method previously described.[7−10,15]

Time Constant Analysis. After being implanted in the rat for 5 h, a DT/MH-functionalized AgFON surface was removed and placed in a flow cell containing bovine plasma to simulate the in vivo environment. Step changes in glucose concentration were made by introduction into the cell of high and low concentrations of glucose dissolved in plasma. The data were processed using PeakFit 4.12 software (Systat Software Inc, Richmond, CA). To remove the varying background in SERS spectra, a fourth-order polynomial was subtracted from the baseline using MATLAB software. The spectra were further preprocessed in PeakFit with linear best-fit baseline correction and Savitsky−Golay smoothing. The amplitude of the Raman bands was obtained by fitting the data to the superposition of the Lorentzian amplitude line shapes. The data were then iteratively fit to an exponential curve to minimize the residuals.

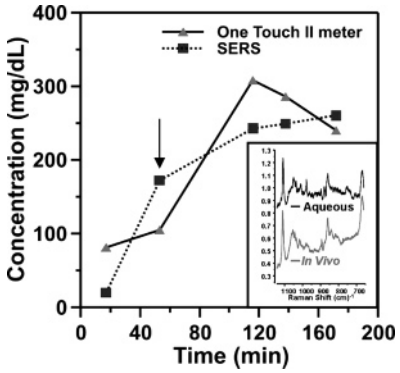

Figure 2. Time course of the in vivo glucose measurement. Glucose infusion was started at t = 60 min. Triangles (▲) are measurements made using One Touch II blood glucose meter, and squares (■) are measurements made using the SERS sensor. Glucose infusion was started at ~1 h, as demarcated by the arrow. The inset shows a typical in vivo spectrum compared to a typical ex vivo spectrum of the same surface. (λ_{ex} = 785 nm, P = 50 mW, t = 2 min).

RESULTS AND DISCUSSION

Comparison of SERS to Electrochemical Measurement. Figure 2 shows the glucose concentration variation in the rat measured using SERS and the One Touch II blood glucose meter with respect to time. The inset shows that autofluorescence is

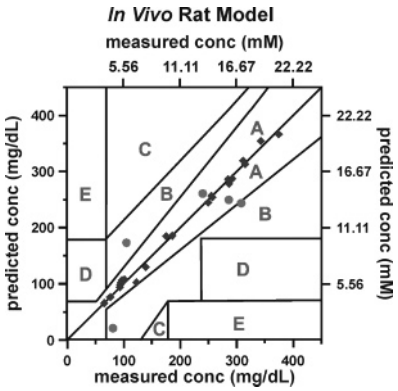

Figure 3. Clarke error grid analysis. Representative data from calibration (◆) and validation (●) plot using a single sensor and a single spot on a DT/MH-functionalized AgFON surface, in vivo. The calibration set was constructed using 21 data points correlated with the commercial glucometer. The validation set utilized 5 data points. (RMSEC = 7.46 mg/dL (0.41 mM), RMSEP = 53.42 mg/dL (2.97 mM), λ_{ex} = 785 nm, P = 50 mW, t = 2 min).

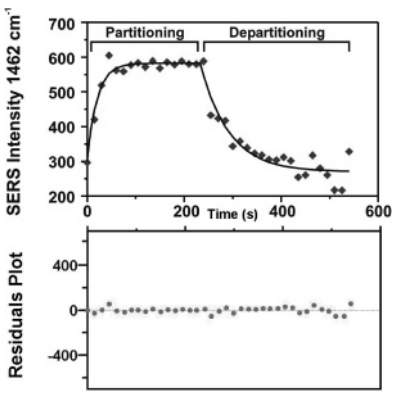

Figure 4. Ex vivo analysis of response time. Real-time SERS response to a step change in glucose concentration after being implanted in a rat for 5 h (above), and a plot of the residuals (below). Glucose was injected at t = 0 s, and the cell was flushed with bovine plasma at t = 225 s. The 1/e time constants were calculated to be 9 s for partitioning and 27 s for departitioning. (λ_{ex} = 785 nm, P = 100 mW, t = 15 s).

not a significant concern if 785-nm excitation is used, as the typical SERS spectra of a DT/MH FON prior to implantation and in vivo show only minor differences, attributable to the change in environment. Both the standard glucometer and the SERS-based measurements effectively tracked the change in glucose concentration. A sharp rise in glucose concentration is detected by both techniques after the start of the glucose infusion (t = 60 min). Figure 3 plots the time-independent data on the Clarke error grid to more precisely gauge the performance of the SERS measurement system.

Error Grid Analysis. The Clarke error grid was developed as a convenient and modality-independent means to compare the accuracy and performance of glucose sensors in the clinically relevant range.[24] The grid is divided into five zones, and predictions within these zones lead to the following: (A) clinically correct measurement and treatment, (B) benign errors or no treatment, (C) incorrect measurements leading to overcorrection of acceptable glucose levels, (D) dangerous failure to detect and treat, and (E) treatments that further aggravate abnormal glucose levels. The majority of measurements from all samples fell within the acceptable range. Figure 3 shows a representative Clarke error grid analysis of a single rodent. The 26 measurements were taken from a single spot on the implanted DT/MH-functionalized AgFON surface. The calibration set was constructed using 21 data points, which were correlated with the commercial glucometer. The validation set utilized the remaining five measurements as independent data points. The sensor had relatively low error (RMSEC = 7.46 mg/dL (0.41 mM) and RMSEP = 53.42 mg/dL (2.97 mM)). These data compare favorably with our previous in vitro results,[8] as well as those of other optically based glucose measurements,[25−28] and with existing detection methods, which have instrument-dependent coefficients of variation of 0.96−26.9% (0.096−2.69 mM, 1.75−49 mg/dL at 10 mM).[29,30]

Time Constant Analysis. Important parameters governing the overall efficacy of a given sensor are its response time, reversibility, and long-term stability. Previous research has demonstrated that FON-based sensors are quite stable with good reversibility under a variety of conditions.[9,16,31,32] The present work shows the DT/M SERS sensor possesses sufficiently rapid response to measure the glucose injection, keeping pace readily with the conventional glucometer. However, the infusion rate used was slow, and there is an inherent lag in the physiological glucose levels, particularly in interstitial fluid. Because it was impossible to rapidly and accurately vary glucose levels in vivo, additional in vitro experiments were conducted to determine whether the sensor is capable of exhibiting response times rapid enough for continuous or semicontinuous monitoring (viz. ≤ the 2-min collection time). This experiment was designed to address concerns about proper functioning of the sensor surface after implantation, such as fouling, and to confirm the results of our previous work.[9] After being implanted in the rat for 5 h, the window was disassembled, and the DT/MH-functionalized AgFON surface was carefully removed with tweezers and immediately placed in a flow cell containing bovine plasma to simulate the in vivo environment. Then, the 1/e time constant response to a step change in glucose concentration was determined (Figure 4**)**. The AgFON surface was exposed to 50 mM glucose in plasma at t = 0 s and then flushed with plasma at t = 225 s. SERS spectra were collected every 15 s (λ_{ex} = 785 nm, P = 100 W). Based upon amplitude calculations for the 1462-cm^{-1} peak, the 1/e time constant was 9 s for partitioning and 27 s for departitioning. These values indicate that glucose binds reversibly to the SERS-active surface and that changes in concentration as rapid as ~30 s can be detected spectroscopically.

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CONCLUSIONS

The automated and continuous monitoring of glucose levels is critically important to the treatment and care of millions of people, particularly in juvenile cases where compliance with a strict medical regimen is often problematic. With further refinements in the system, viz. instrumentation, optics, and surface, the SERS-based glucose sensor has the potential to replace conventional personal and point-of-care assays. We hope to expand the number and type of metabolic analytes that can be measured with this technique by chemically tailoring the partitioning layer. We are presently using DT/MH FONs in the detection of lactate in serum and plan to develop a SERS sensor capable of simultaneous multianalyte detection. Furthermore, we are actively expanding the concept of chemical modification of the SERS surfaces to include materials other than SAMs. For example, recent results indicate that surfaces prepared by atomic layer deposition show great promise in promoting the stability of the surfaces, while providing chemically selective surfaces.[33] Finally, the development of a transdermal implantable sensor, where both the excitation and Stokes-shifted photons pass directly through the skin, will minimize the invasiveness of the procedure. Looking to the future, we predict that the functionalized SERS sensor approach will have important applications in both opening up new areas of fundamental research and treatment and care of diabetics.

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