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Chapter 8

Surface-Enhanced Raman Sensing: Glucose and Anthrax

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This chapter outlines the use of surface-enhanced Raman spectroscopy (SERS) in the development of biological sensors for the detection of anthrax and glucose. In both cases, Ag film over nanosphere (AgFON) surfaces were used as the sensing platform. The localized surface plasmon resonance (LSPR) of AgFON surfaces was tuned to maximize the SERS signal for near infrar-red (NIR) excitations. A harmless analog of *Bacillus anthracis*, namely *Bacillus subtilis*, was quantitatively detected using SERS on AgFON surfaces. Calcium dipicolinate, an important biomarker for bacillus spores, was successfully measured with a limit of detection well below the anthrax infectious dose of 10^4 spores in 11 minutes. For glucose detection, a mixed self-assembled monolayer (SAM) consisting of decanethiol (DT) and mercaptohexanol (MH) was immobilized on an AgFON surface to bring glucose within the zone of the localized electromagnetic field. Complete partitioning and departitioning of glucose was demonstrated. Furthermore, quantitative detection *in vitro* and *in vivo* was achieved.

Introduction

The rapid and accurate identification of biomoleclues is vital for first responders in the event of a biological attack, as well as for patients with a need to monitor chronic or acute conditions. In this chapter, we outline the use of surface-enhanced Raman spectroscopy (SERS) for the detection of calcium dipicolinate (CaDPA), a biomarker for anthrax, and glucose, an indicator for diabetes.

Anthrax is an infection disease caused by *Bacillus anthracis* that requires medical attention within 24-48 hours after initial inhalation of more than 10^4 *B. anthracis* spores (*1*). Therefore, the rapid detection of *B. anthracis* spores in the environment prior to infection is an extremely important goal for human safety. Structurally, a spore consists of a central core cell surrounded by various protective layers. CaDPA is located in these protective layers and accounts for ~10% of the spore's dry weight (*2*) making it is a useful biomarker for bacillus spores (*3*).

Diabetes mellitus is a metabolic disorder where the body either fails to produce insulin or does not respond to insulin, which mediates the uptake of glucose. Failure to regulate glucose levels within tight limits can lead to severe secondary health complications including blindness, kidney disease, nerve damage, and malfunctions related to the circulatory system (*4, 5*). Currently available blood glucose meters are not suitable for continuous monitoring and suffer from low patient compliance, risk of false positives due to interferents, and instability caused by finite protein life cycle. Developing a sensor that measures glucose directly and continuously, in the presence of interfering analytes, will have an enormous impact on the long-term health of diabetics.

We have utilized SERS to design biosensors for both anthrax and glucose. The SERS-based sensor has many advantages: high sensitivity, rapid detection capabilities, and unique spectral fingerprints for detection in the presence of interfering analytes. Normal Raman spectroscopy (NRS), is a very selective technique because every molecular species (e.g. glucose and fructose) has a unique vibrational signature. The limitation of NRS, however, is its weak signal intensity. Higher signal intensity can be achieved by SERS, in which the analyte of interest is brought within a few nanometers of a roughened metal surface, amplifying the signal by 10^6-10^8 and in some cases as high as 10^{15}.

SERS signals can be optimized by tuning the optical properties, known as localized surface plasmon resonance (LSPR), of metal nanostructured SERS-active surfaces to match the experimental parameters. SERS signals are most intense when the LSPR maximum wavelength lies between the excitation wavelength and energy of the Raman scattered photons (*6*). SERS-based sensors utilize Ag film over nanosphere (AgFON) surfaces with highly tunable plasmons, which are optimized to achieve maximum SERS signal. AgFON surfaces are fabricated by depositing silver on hexagonally close-packed

polystyrene spheres. The tunability of the LSPR can be easily achieved by changing the sphere diameter (D), as well as the silver metal thickness (d_m).

While CaDPA biomarker in *B. subtilis* spores has an affinity towards the rough silver surface required for SERS detection, many important molecules (e.g. glucose) have low or non-existent binding affinity towards a silver surface (*7*). The work presented herein demonstrates quantitative glucose detection by functionalizing the SERS-active surface with a self-assembled monolayer (SAM). The SAM partitions glucose and concentrates it near the surface of the AgFON (*7-9*). We use a mixed SAM (Figure 1) consisting of decanethiol (DT) and mercaptohexanol (MH) whose dual hydrophobic and hydrophilic properties make it ideal for partitioning glucose (*10*). Although the exact mechanism of DT/MH SAM partitioning has not been well characterized, a space filling computer model shows that combining DT and MH creates a pocket for the analyte. It is hypothesized that glucose partitions into this compartment and approaches closer to the surface than was possible with SAMs used previously (*7-9*).

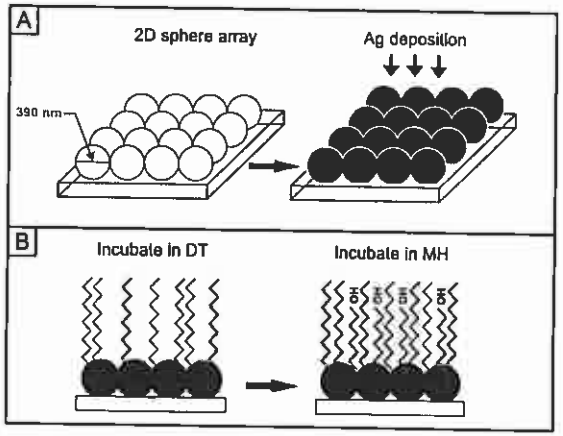

Figure 1: (A) Schematic diagram representing AgFON fabrication. (B) Formation of the DT/MH self-assembled monolayer on the AgFON surface.

Experimental Section

Spore Samples

B. subtilis was purchased from the American Type Culture Collection (Manassas, VA). Spore cultures were cultivated by spreading the vegetative

cells on sterile nutrient agar plates (Fisher Scientific), followed by incubating at 30 °C for 6 days. The cultures were washed from the plates using sterile water and centrifuged at 12000 g for ten minutes. The centrifuging procedure was repeated five times. The lyophilized spores were kept at 2-4 °C prior to use. Approximately 1 gram of sample was determined to contain 5.6×10^{10} spores by optical microscopic measurements. The spore suspension was made by dissolving spores in 0.02 M HNO_3 solution and by sonicating for 10 minutes. Calcium dipicolinate was prepared from DPA and calcium hydroxide according to the method of Bailey and co-workers (*2*).

AgFON Substrate Fabrication

Glass and copper substrates for anthrax detection and glucose detection respectively were pretreated as described previously (*10, 11*). Approximately 2 μL of the nanosphere suspension (4% solids) was drop-coated onto each glass substrate and 10 μL of the nanosphere suspension was drop-coated onto each copper substrate and allowed to dry in ambient conditions. The metal films were deposited in a modified Consolidated Vacuum Corporation vapor deposition system with a base pressure of 10^{-6} Torr. The deposition rates for each film (10 Å/sec) were measured as described previously (*10*). AgFON surfaces were stored in the dark at room temperature prior to use.

For glucose detection, AgFON surfaces were first incubated in 1 mM DT in ethanol for 45 min and then transferred to 1 mM MH in ethanol for at least 12 h (Figure 1B). The SAM-functionalized surfaces were then mounted into a small-volume flow cell for SERS measurements.

SERS Apparatus

A titanium-sapphire laser (CW Ti: Sa, model 3900, Spectra Physics, Mountain View, CA) pumped by a solid-state diode laser, λ_{ex} 532 nm (model Millenia Vs, Spectra Physics) was used to generate λ_{ex} of 750 or 785 nm as described previously(*10, 11*). For the detection of spores, all the measurements were performed in ambient conditions. For the detection of glucose, a small-volume flow cell was used to control the external environment of the AgFON surfaces.

LSPR Reflectance Spectroscopy, quantitative multivariate analysis and time constant analysis was performed as described in our papers (*10, 11*).

Results and Discussion

Extraction of CaDPA from Spores

CaDPA was extracted from spores by sonicating in 0.02 M HNO_3 solution for 10 minutes. This concentration of the HNO_3 solution was selected because of its capability of extraction and benign effect on the AgFON SERS activity. The sonication procedure was performed because no SERS signal of CaDPA was observed from the spore solution prior to sonication. To test the efficiency of this extraction method, a 3.1×10^{-13} M spore suspension (3.7×10^4 spores in 0.2 μL, 0.02 M HNO_3) was deposited onto a AgFON surface (D = 600 nm, d_m = 200 nm). The sample was allowed to evaporate for less than one minute. A high signal-to-noise ratio (S/N) SER spectrum was obtained in 1 minute (Figure 2A). For comparison, a parallel SERS experiment was conducted using 5.0×10^{-4} M CaDPA (Figure 2B). The SER spectrum of *B. subtilis* spores is dominated by bands associated with CaDPA, in agreement with the previous Raman studies on bacillus spores (*12, 13*). The peak at 1050 cm^{-1} in Figure 2A is from the symmetrical stretching vibration of NO_3^- (*14*). Because of its prominence, this peak is used as an internal standard to reduce sample to sample deviations.

Temporal Stability of AgFON and SAM-functionalized AgFON Substrates

An ideal detection system should run unattended for long periods of time, require infrequent maintenance, and operate at low cost. Previous work has demonstrated that bare AgFON surfaces display extremely stable SERS activity when challenged by negative potentials in electrochemical experiments (*15*) and high temperatures in ultrahigh vacuum experiments (*16*). Moreover, as shown previously by spectroscopic and electrochemical measurements, SAMs adsorbed on metal FON substrates remain stable for 3 to 10 days depending on the metal (*9*). In this work, the temporal stability of AgFON and DT/MH SAM-functionalized AgFON surfaces was studied over a period of 40 and 10 days, respectively.

SER spectra of 4.7×10^{-14} M spores (5.6×10^3 spores in 0.2 μL, 0.02 M HNO_3), well below the anthrax infectious dose of 10^4 spores, were captured on AgFON surfaces of different ages. Both the CaDPA spectral band positions and intensity patterns remained constant over the course of 40 days (*11*). Furthermore, SER spectra of the DT/MH SAM on AgFON surfaces stored in bovine plasma were acquired daily over a period of 10 days. Spectral position of

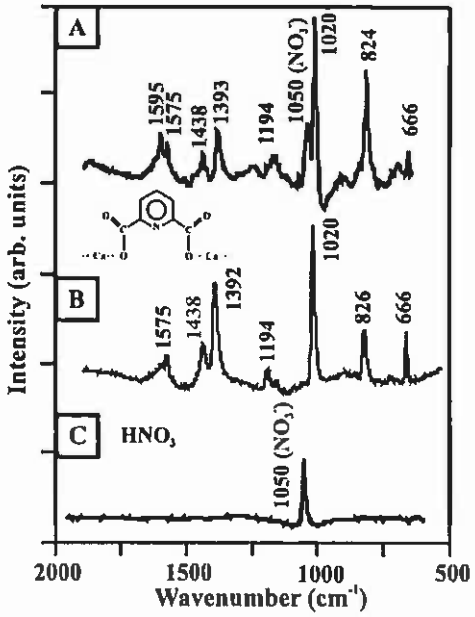

Figure 2. (A) SER spectrum of 3.1 × 10^{-13} M spore suspension (3.7 × 10^{4} spores in 0.2 μL, 0.02 M HNO_3) on a AgFON substrate. (B) SER spectrum of 5.0 × 10^{-4} M CaDPA. (C) SER spectrum of 0.2 μL 0.02 M HNO_3. λ_{ex} = 750 nm, laser power = 50 mW, t = 1 min, D = 600 nm, d_m = 200 nm. Reproduced with permission from ref. 11. Copyright 2005 J. Am. Chem. Soc.

the C-C stretching vibrational band at 1119 cm^{-1} remained constant and the intensity decreased by only 2%, which can be attributed to rearrangments in the SAM (*10*). This indicates AgFONs' suitability for potential field sensing applications, as well as *in vivo* detection when functionalized with a SAM.

Adsorption Isotherm and LOD for Bacillus Spores on AgFON Surfaces

The quantitative relationship between SERS signal intensity and spore concentration is demonstrated in Figure 3A. Each data point represents the average intensity at 1020 cm^{-1} from three samples with the standard deviation shown by the error bars. At low spore concentrations, the peak intensity increases linearly with concentration (Figure 3A inset). At higher spore concentrations, the response saturates as the adsorption sites on the AgFON

substrate become fully occupied. Saturation occurs when the spore concentrations exceed ~ 2.0 × 10^{-13} M (2.4 × 10^{4} spores in 0.2 μL, 0.02 M HNO_3).

To be practical for long-term health and safety monitoring, a SERS-based detection system has to be capable of detecting less than the life-threatening dose of a pathogen in real or near-real time. In this study, the LOD is defined as the concentration of spores for which the strongest SERS signal of CaDPA at 1020 cm^{-1} is equal to three times the background SERS signal within a one-minute acquisition period. The background signal refers to the SERS intensity from a sample with a spore concentration equal to zero, which is calculated to be the intercept of the low concentration end of the spore adsorption isotherm (Figure 3A). Although lower detection limits can be achieved using longer acquisition times, these parameters are reasonable for high throughput, real-time, and on-site analysis of potentially harmful species. The LOD for *B. subtilis* spores, evaluated by extrapolation of the linear concentration range of the adsorption isotherms (Figure 3A inset), is found to be 2.1 × 10^{-14} M (2.6 × 10^{3} spores in 0.2 μL, 0.02 M HNO_3). It should be noted that previously published NRS studies of anthrax detection were 200,000 times less sensitive and required eight times more laser power (*12*). Similarly, previous SERS studies via the CaDPA biomarker were 200 times less sensitive and required three times more laser power (*13*) than the results demonstrated herein.

To determine the adsorption capacity of extracted CaDPA on an AgFON, the Langmuir adsorption isotherm was used to fit the data (*17, 18*):

$$\theta = \frac{I_{1020}}{I_{1020,\max}} = \frac{K_{spore} \bullet [spore]}{1 + K_{spore} \bullet [spore]} \qquad (1)$$

$$\frac{1}{I_{1020}} = \frac{1}{K_{spore} \bullet I_{1020,\max}} \bullet \frac{1}{[spore]} + \frac{1}{I_{1020,\max}} \qquad (2)$$

where θ is the coverage of CaDPA on the AgFON; $I_{1020\ max}$ is the maximum SERS signal intensity at 1020 cm^{-1} when all the SERS active sites on the AgFON are occupied by CaDPA; [*spore*] is the concentration of spores (M); and K_{spore} is the adsorption constant of CaDPA extracted from spores on AgFON (M^{-1}). From equation 2, K_{spore} is calculated from the ratio between the intercept and the slope. Slope and intercept analyses of the linear fit (Figure 3B) leads to the value of the adsorption constant K_{spore} = 1.7 × 10^{13} M^{-1}.

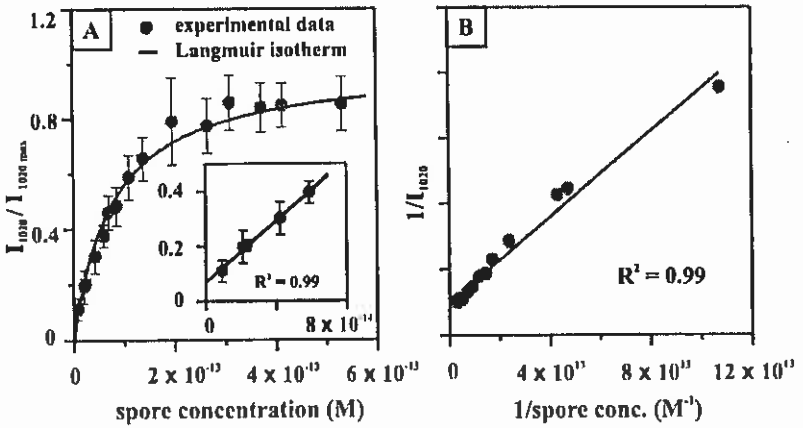

Figure 3. (A) Adsorption isotherm for B. subtilis spore suspension onto a AgFON substrate. I_{1020} was taken from SER spectra that correspond to varying spore concentrations in 0.2 μL, 0.02 M HNO_3 on AgFON surfaces. λ_{ex} = 750 nm, laser power = 50 mW, t = 1 min, D = 600 nm, and d_m = 200 nm. A Langmuir curve was generated using eq 1 with $K_{spore} = 1.3 \times 10^{13}\ M^{-1}$. The inset shows the linear range that is used to determine the LOD. Each data point represents the average value from three samples. Error bars show the standard deviations. (B) Adsorption data fit with the linear form of the Langmuir model, eq 2. The slope and intercept values are used to calculate the adsorption constant. Reproduced with permission from ref. 11. Copyright 2005 J. Am. Chem. Soc.

Use of Field-Portable Raman Spectrometer for Anthrax Detection

The final goal of this project was to demonstrate the use of SERS as a field-portable screening tool by using a compact Raman spectrometer. Many field-sensing applications require the portability and flexibility not available from conventional laboratory scale spectroscopic equipment. As a first step in this direction, the Raman spectrum from 10^4 *B. subtilis* spores dosed onto a one-month-old AgFON substrate was readily acquired using a commercially available portable Raman instrument. A high S/N spectrum was achieved within five seconds (Figure 4A). The SERS peak positions and intensity pattern for the spore sample was similar to those of CaDPA recorded utilizing the same device (Figure 4B). This is the first example of using a compact, portable Raman spectrometer for the detection of bacillus spores. By coupling the portability and ease of use of this type of device with the molecular specificity and spectral sensitivity inherent to SERS, we open a range of possibilities in the area of detecting bioagents and other chemical threats. In practical field applications of the detection method described, there might be difficulties in collecting *B. anthracis* spores out of the air and dissolving them into a small liquid volume. Most sensor modalities must face this problem.

Reversibility of DT/MH AgFON Glucose Sensor

While CaDPA binds directly to a bare AgFON surface, some molecules such as glucose do not have an affinity to either Ag or Au and need a partition layer to bring them closer to the surface. For glucose detection, we have demonstrated the viability of a DT/MH-functionalized SERS-based sensor. An implantable glucose sensor must successfully monitor the fluctuation of glucose continuously throughout the day. This can only be accomplished if the sensor can completely partition and departition glucose. To simulate real-time sensing, 0 and 100 mM aqueous glucose solutions (pH ~7) were alternately introduced into a flow cell containing the DT/MH-modified AgFON in 20-min intervals without flushing the sensor between measurements. Figures 5A, 5B, and 5C show the first three steps of the pulsing experiment. Figures 5D is the difference spectra that demonstrates partitioning of glucose into the DT/MH functionalized SAM with glucose features at 1461, 1371, 1269, 1131, 916, and 864 cm^{-1}. For comparison to the SERS spectra, Figure 5F shows the normal Raman spectrum of an aqueous saturated glucose solution. Peaks at 1462, 1365, 1268, 1126, 915, and 850 cm^{-1} correspond to crystalline glucose peaks (*19*). The related literature has shown that SERS bands can shift up to 25 cm^{-1} compared to normal Raman bands of the same compound (*20*). Therefore, the features in the difference spectra correspond to the glucose peaks in the normal Raman spectrum of glucose (Figure 5F). Departitioning of glucose is demonstrated by the difference

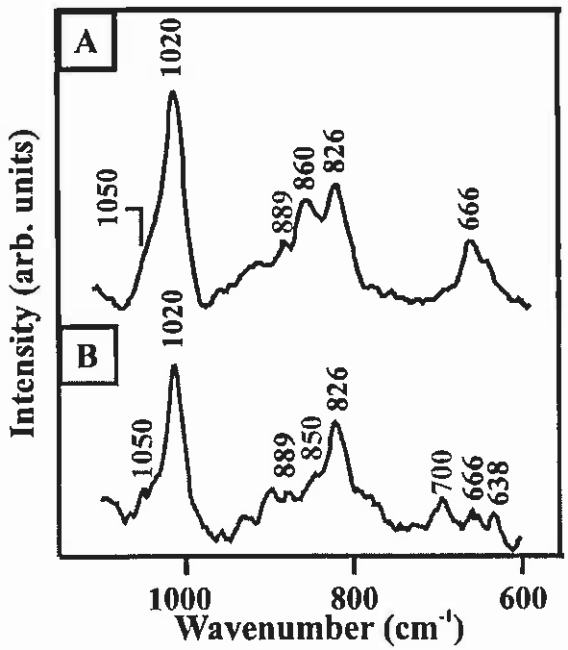

Figure 4. SER spectra obtained by portable Raman spectrometer. (A) SER spectrum of 8.3 × 10^{-14} M spore suspension (1.0 × 10^{4} spores in 0.2 μL, 0.02 M HNO_3) on 30-day old AgFON. (B) SERS spectrum of 10^{-4} M CaDPA in 0.2 μL 0.02 M HNO_3 on 30-day old AgFON substrate. λ_{ex} = 785 nm, laser power = 35 mW, t = 5 sec, resolution = 15 cm^{-1}, D = 600 nm, and d_m = 200 nm. Reproduced with permission from ref. 11 Copyright 2005 J. Am. Chem. Soc.

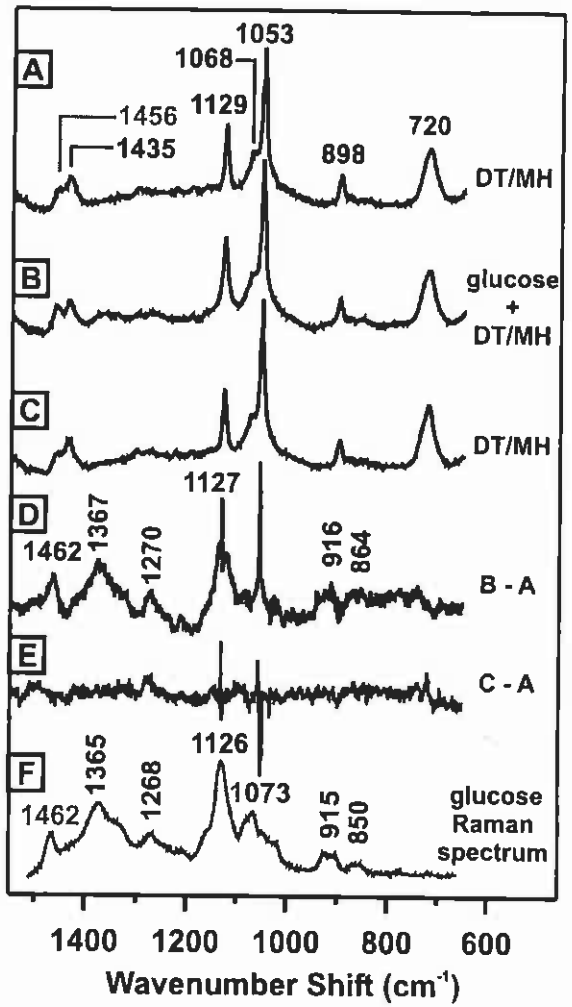

Figure 5. SER spectra demonstrating reversibility of DT/MH functionalized SAM. λ_{ex} = 532 nm, laser power = 10 mW, t = 20 min, pH ~7. (A), (B), and (C) show the first three steps in the pulsing spectrum where 0 and 100 mM aqueous glucose solutions (pH~7) were alternately introduced into a flow cell. (D) shows a difference spectrum demonstrating partitioning. (E) shows a difference spectrum demonstrating departitioning. The normal Raman spectrum of aqueous saturated glucose solution is shown in (F).

spectra shown in Figure 5E. The absence of glucose spectral features clearly demonstrates complete departitioning of glucose. The spectra were normalized by using the nitrate peak at 1053 cm^{-1} as an internal standard to minimize laser power fluctuations. These experiments clearly demonstrate that the DT/MH-functionalized AgFON is a reversible sensing surface that can completely partition and departition glucose.

Time Constant of DT/MH AgFON Glucose Sensor

In addition to reversibility, an implantable glucose sensor must also partition and departition on a reasonable time scale. The real time response was evaluated by calculating the 1/e time constants for partitioning and departitioning. The experiments were conducted in bovine plasma to simulate the *in vivo* environment.

A DT/MH-functionalized AgFON was first incubated in bovine plasma for ~5 hours. The AgFON surface was then placed in a flow cell and a solution containing 50 mM glucose in bovine plasma was introduced at t = 0 to observe partitioning. Departitioning was evaluated by injecting 0 mM glucose in bovine plasma into the flow cell at t = 225 s. Throughout the experiment, SER spectra were collected continuously at 15 s intervals.

The amplitude of the peak at 1462 cm^{-1} was obtained by fitting the spectra to the superposition of three Lorentzian lineshapes and was plotted versus time (Figure 6). The plot was fitted with an exponential curve, yielding the following 1/e time constants: 28 s for partitioning and 25 s for departitioning (*10*). These experiments demonstrate that partitioning and departitioning occur in less than 1 minute, making the SERS based sensor a potential candidate for implantable, continuous sensing.

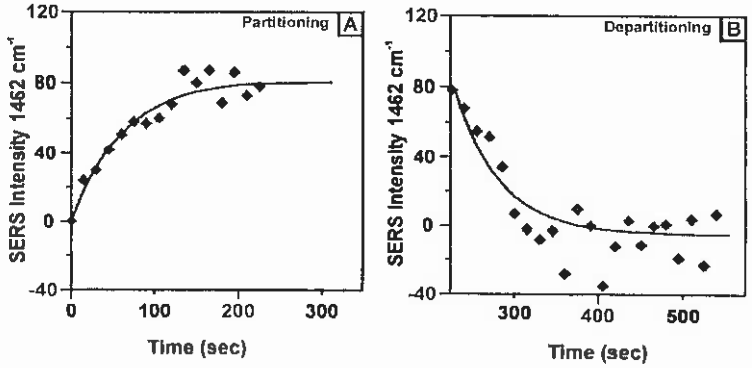

Figure 6. Real-time response to step change in glucose concentration. Partitioning (A) and departitioning (B) of glucose is demonstrated with with λ_{ex} = 785 nm, laser power = 100 mW, t = 15 s. The 1/e time constants were calculated to be 28 s for partitioning and 25 s for departitioning.

Calibration Curve of DT/MH AgFON Glucose Sensor

Futhermore, an ideal glucose sensor must be able to detect glucose in the clinically relevant range 10-450 mg/dL (0.56-25 mM), under physiological pH and in complex media. The DT/MH-functionalized SAM was placed in a flow cell and incubated for 2 minutes with glucose (10 – 450 mg/dl) in filtered bovine plasma. Bovine plasma was used to simulate the *in vivo* environment that the sensor will be exposed to when it is implanted under the skin in interstitial fluid. SER spectra were collected using multiple samples and multiple spots with a near-infrared laser source. Partial least squares leave-one-out (PLS-LOO) analysis was used to construct a calibration model with 92 independent spectral measurements of known glucose concentrations based on 7 latent variables. The resulting calibration model and concentration predictions are presented on a Clarke error grid (Figure 7).

The Clarke error grid is a standard for evaluating the reliability of glucose sensors in the clinically relevant concentration range (0-450 mg/dL) (*21*). Data

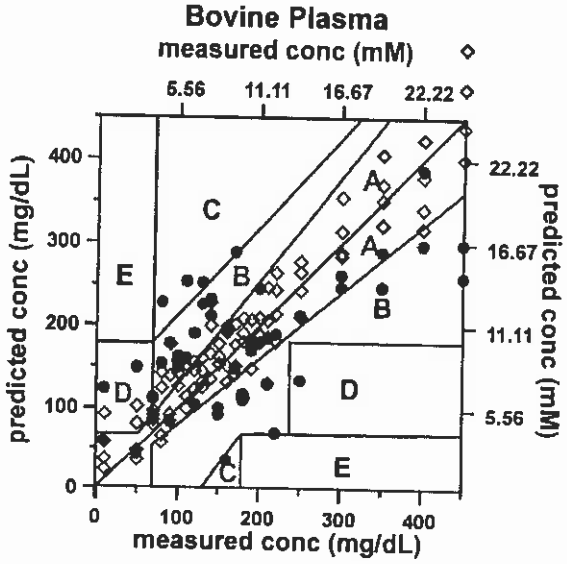

Figure 7. Calibration (◇) and validation (●) plots of glucose in bovine plasma on Clarke error grid. Calibration plot was constructed using 92 data points and validation plot was constructed using 46 data points. Sample was incubated for 2 min in glucose concentrations ranging from 10 – 450 mg/dl with λ_{ex} = 785 nm, laser power = 10-30 mW, t= 2 min. RMSEC = 34.3 mg/dL (1.9 mM) and RMSEP = 83.16 mg/dL (4.62 mM). Reproduced with permission from ref. 10

points that fall in the A and B ranges are acceptable values. Values outside the A and B range indicate potential failure to detect blood glucose levels properly, usually resulting in erroneous and even fatal diagnosis.

The PLS analysis yielded a root mean square error of calibration (RMSEC) of 34.3 mg/dL (1.90 mM) with 98% of the data falling in the A and B region of the Clarke error grid. The root mean square error of prediction (RMSEP) was calculated to be 83.16 mg/dL (4.62 mM) with 85% of the validation points falling in the A and B range of the Clarke error grid (*10*). Overall, the results show that the DT/MH-modified AgFON glucose sensor is capable of making accurate glucose measurements in the presence of interfering analytes.

Calibration Curve for *in Vivo* DT/MH AgFON Glucose Measurement

Finally, we demonstrated the first successful detection of glucose *in vivo* with an implantable SERS sensor using a rat animal model (*22*). To collect SER spectra, a metal frame containing a glass window was placed along the midline of the back of a Sprague-Dawley rat. A DT/MH-functionalized AgFON surface supported on a Cu mesh was positioned between the skin and the window such that the substrate was in contact with the interstitial fluid. An aliquot of glucose (1 g/mL in saline) was injected in the rat to artificially vary the blood glucose level. An aliquot of blood was drawn from the rat to measure the blood glucose level using the One Touch II blood glucose meter. SER spectra were acquired through the window and showed pronounced Raman band characteristic of the SAM (*22*). Additional bands were observed compared to the spectra collected in the bovine plasma model; however, the Raman bands were not obscured by autofluorescence because near infra-red excitation was used. All SER spectra were analyzed using PLS-LOO and represented on the Clarke error grid. In Figure 8, 21 data points were used to build the calibration model and five data points were used to validate the model. All the data points in both calibration and validation fall in the A and B range of the Clarke error grid with RMSEC = 7.46 mg/dL and RMSEP = 53.42 mg/dL. This is the first report of *in vivo* glucose detection using SERS.

Conclusion

We have successfully demonstrated the applicability of SERS for biological sensing. We have targeted analytes such as CaDPA, a marker for anthrax, as well as, glucose, which plays a crucial role in evaluating metabolic health. In brief, we have shown that SERS is a highly sensitive and selective method allowing rapid quantitative detection of both analytes. In addition, we have

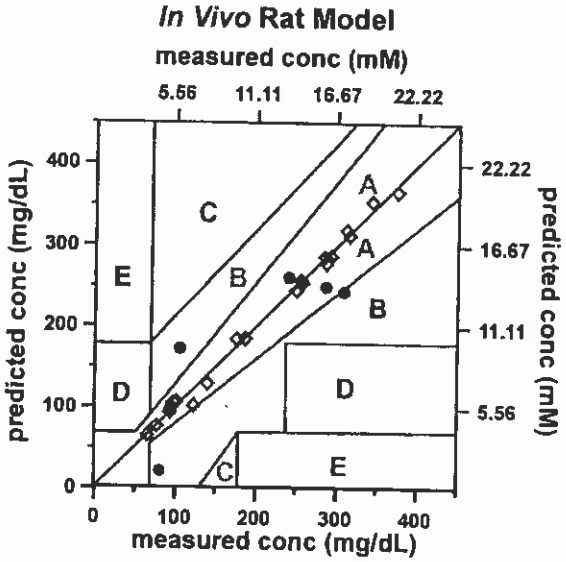

Figure 8. Glucose calibration (◇) and validation (●) plot using a single substrate and a single spot on a DTMH functionalized AgFON on a mesh. PLS calibration plot was constructed using 21 data points and validation plot was constructed using 5 data points taken over a range of glucose concentrations (10 - 450 mg/dL) in vivo (rat) with λ_{ex}=785nm, laser power = 50 mW, t = 2 min. RMSEC = 7.46 mg/dL (0.41 mM) and RMSEP = 53.42 mg/dL (2.97 mM).

demonstrated that SERS-active surfaces such as AgFONs are suitable for long term use in the field and as implantable devices in the body. Finally, we demonstrate for the first time the use of a portable Raman spectrometer for anthrax detection and quantitative *in vivo* glucose measurements. Future work will focus on exploring various surface chemistries to improve selective binding of CaDPA to a SERS-active surface, as well as SAMs that minimize spectral overlap with glucose. We will also incorporate a miniaturized spectrometer and a fiber optic probe for transcutaneous measurements of glucose, and perform long-term *in vivo* studies.

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