DEVELOPMENT OF A LASER-BASED DETECTION SYSTEM FOR WATER-BORNE PATHOGENS

By

Geoffrey B. Smith
Kevin H. Oshima
Biology Department
New Mexico State University

TECHNICAL COMPLETION REPORT

Account Number 01345670

June 2004

New Mexico Water Resources Research Institute

in cooperation with the

Department of Biology
New Mexico State University

The research on which this report is based was financed in part by the U.S. Department of the Interior, Geological Survey, through the New Mexico Water Resources Research Institute.

DISCLAIMER

The purpose of the Water Resources Research Institute technical reports is to provide a timely outlet for research results obtained on projects supported in whole or in part by the institute. Through these reports, we are promoting the free exchange of information and ideas, and hope to stimulate thoughtful discussions and actions that may lead to resolution of water problems. The WRRI, through peer review of draft reports, attempts to substantiate the accuracy of information contained in its reports, but the views expressed are those of the authors and do not necessarily reflect those of the WRRI or its reviewers. Contents of this publication do not necessarily reflect the views and policies of the Department of the Interior, nor does the mention of trade names or commercial products constitute their endorsement by the United States government.

ABSTRACT

Procedures and experiments described in this report were aimed at determining the feasibility of using surface-enhanced Raman spectroscopy (SERS) to detect water-borne microbial pathogens. Two laser sources were used to interrogate the biological samples with wavelength maxima of 532 nm and 632 nm, the Nd:YVO_4 and HeNe lasers, respectively. Representative Gram-negative (*Escherichia coli* and *Salmonella enterica*) and Gram-positive (*Bacillus megaterium*, *B. globigii* and *B. subtilis*, a surrogate for the pathogen, *B. anthracis*) bacteria were assayed, and a surrogate for enteric viruses was also used (the bacteriophage, PP7). Two approaches were used to develop this laser-based detection system: 1) nano-particle gold-labeled antibodies were used to distinguish the *Salmonella* pathogen from the other bacteria and 2) nano-particle silver was used without antibody to directly assay cell surface differences to differentiate the test microbes.

The laser assay using the antibody approach was successful in differentiating pathogen from non-pathogen, in spite of the cross-reactivity of the antibody. The silver colloid direct detection approach differentiated between *Bacillus* species but no interpretable signal was measurable when using either *E. coli* or PP7 virus. The positive results were obtained within a 5-minute period, indicating the potential for real-time detection of some water-borne pathogens.

TABLE OF CONTENTS

List of Figures

INTRODUCTION

There have been numerous analytical approaches described to detect microbial pathogens in an effort to decrease the time of analysis and detection limit. Probably the most common approach that has yielded the most promising results is the polymerase chain reaction (PCR), and more recently, real-time PCR. Using an air-heated thermal cycler, DePaulo (2002) have detected the water-borne pathogen *Vibrio parahemolyticus* in oysters within one hour of sampling by directly amplifying the fluid inside the oyster, "the liquor." Though detection limits were relatively high, the speed of analysis allowed the FDA employees to make oyster harvesting dependent on water quality in realtime. PCR has been miniaturized and developed as a continuous-flow system on a microchip (Kopp et al. 1998), and thus this DNA amplification-based approach shows continued promise toward solving real-world microbial detection issues such as detection of *B. anthracis* (Qi et al. 2001).

Nevertheless, limitations on PCR applications included the need to prepare the sample for analysis. At the least, the target cells need to be lysed, and, in the presence of contaminated environmental samples, the released DNA must be purified before amplification. One of many analytical alternatives for microbial detection, is the use of Surface-Enhanced Raman Spectroscopy (SERS) to interrogate biological surfaces without the need to lyse the cellular target. The SERS technique employs low-power lasers (we use 10 – 300 mW), which, in combination with nanoparticle colloids, allows for logarithmic increases in signal (Fleischmann et al. 1974). Recently, physicists at New Mexico State University have discovered and patented a new SERS technology based on fractals that amplifies the SERS signal by another 3 to 6 log orders of magnitude (Kim et al. 1999). The current work funded by the New Mexico WRRI was aimed at developing this SERS-based system toward detecting and differentiating among bacterial and viral pathogens representative of water-borne pathogens. Applications of SERS for use in the detection of biological materials as well as its application to quantitative assay with biological molecules on metal colloids has been carried out to a limited extent (e.g., Ni et al. 1999).

The emphasis of this work was toward developing a real-time detection system using high target numbers (ca 10^7). Future work will be aimed at increasing the sensitivity of the assay and increasing the number of detectable targets.

METHODS

Microbiology and Immunology

Pure cultures of *Salmonella enterica* (NMSU strain #128), *Escherichia coli* (NMSU strain #150), *Bacillus megaterium* (NMSU strain # 61), *B. globigii* (English Strain # 8058) and *B. subtilis* var. niger (EPA strain) were grown under the following standardized conditions: 17 hours at 30ºC and 150 rpm in 3 mL of nutrient broth. The cultures were enumerated on nutrient agar plates. The *E. coli* strain was verified to be resistant to ampicillin and tetracycline, and the *S. enterica* and the *Bacillus* species were verified to be sensitive to both antibiotics. The PP7 virus was harvested from plaques formed after infection of the bacterial host, *Pseudomonas auruginosa*.

Anti-Salmonella antibody (CSA, generated in goat) was purchased from KPL, Inc. (Gaithersberg, MD) and was used as the primary antibody. The secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA) and is an anti-goat antibody labeled with 12 nm colloidal gold. In order to test the direct use of a single antibody, the Salmonella antibody was conjugated to 20 nm colloidal gold based on the conjugation method described by Slot and Geuze (1985). Briefly, the pH of the colloidal gold sols was raised to pH 9 with 0.5 M NaOH, and, while 8 mL of the gold sols (0.1% $HAuCl_4$, Sigma Chemical Co.) were being stirred, 0.75 mL of antibody was added so the colloidal gold exceeded the saturation point by 10%. After 5 minutes, bovine serum albumin was added to give 0.2% concentration, and the mixture was spun for 30 minutes at 30,000 rpm. After removing the supernatant, the red-colored, diffuse band at the bottom of the tube was retrieved, washed and resuspended in distilled water. This gold-conjugated antibody was then used as described below.

Bacterial cells were exposed to antibodies or were directly irradiated by laser in the following manner. After 17 hours of growth, the cultures absorbance was taken at 560 nm, the cells were pelleted, washed with pH 7.5 PBS and resuspended in variable volumes of PBS so that each cell type had an absorbance of 0.5 O.D (ca 10^8 / mL). The cells were combined in a 1:1 ratio with the antibody to give an antibody dilution that ranged from 1:10 (for the primary conjugated antibody) to 1:50 (for the secondary conjugated antibody), and were incubated for one hour at 37ºC. The cells were then centrifuged, washed three times and resuspended in sterile water and stored at 4ºC until use in the laser assay. If a secondary antibody was used, an additional incubation of one hour was carried out, followed by washes and resuspension in sterile water.

The presence of the antibody bound to the cell was assayed for using traditional enzyme-linked immunosorbent assay (ELISA) by adding antibody specific for the secondary gold antibody, which was labeled with phosphatase and assayed for by adding p-nitrophenol and monitoring color development on a plate reader at 410 nm). Transmission electron microscopy was used to detect the presence of gold-labeled antibody on cell surfaces.

Laser Spectroscopy

Laser spectroscopy was done in collaboration with R. Montoya in Dr. Robert Armstrong's lab in the NMSU Physics Department. The following laser sources were used: red HeNe at 632.8 nm, using 20-40 mW, or the green Nd:YVO_4 at 532 nm, using 70-300 mW. The sample was exposed to the laser source from three min to one hour, and the sample was irradiated while in a 1 mL polystyrene cuvette. After laser irradiation of the sample, the laser pump wavelength was filtered of source light, and the signal was collected by an Acton Spectra Pro 275 grating spectrometer connected to a photomultiplier tube (PMT). The PMT housing unit had two connections, one for the high voltage input, and the other for the signal output and the signal was sent though this output to an A/D converter. The data acquisition system included the DAQ- 801/802 interface and the data were analyzed by MATLAB software written by R. Montoya for this application.

For cells that had been pretreated with gold-labeled antibodies, 0.2 mL samples of antibody-treated and washed cells were combined with 1 mL of distilled water and the sample was irradiated. For the direct assay (using cells without antibody), 0.2 mL of cells were added to the cuvette followed by 1 mL of silver sols and 0.2 mL of 1 M NaOH. These suspensions were then exposed for 3 minutes to the laser as described above.

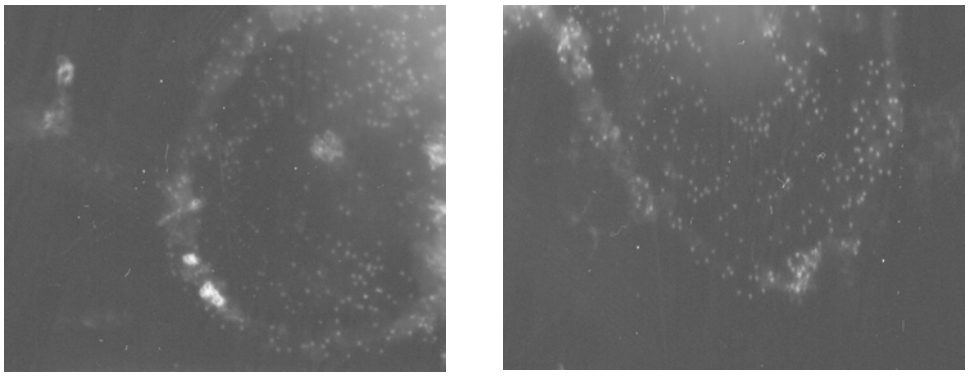
RESULTS

Antibody-based Assays

A gold-labeled secondary antibody against *Salmonella* was used as the basis for three detection assays: 1) traditional ELISA, 2) transmission electron microscopy and 3) the SERS-based assay. The ELISA test resulted in the following absorbances: (410nm) for the target *Salmonella enterica*, and the non-targets *E. coli* and *Bacillus globigii*: 0.97 (0.01), 0.67 (0.04) and 0.46 (0.05) [(Standard Deviation)]. The absorbance in the enzyme-linked assay is related to the number of cell targets present, and thus the ELISA results show that the antibody is specific for *Salmonella*, but that there is significant cross-reaction with non-target species. Similar results were observed when examining the cells by transmission electron microscopy after exposure to the gold-labeled secondary antibody (Figure 1). In contrast to these two traditional detection approaches, the SERS assay exhibited a clear distinction between target *Salmonella* and non-target *E. coli* (Figure 2). It is very interesting to note that, in spite of significant nonspecific binding of the gold-labeled antibody to *E. coli* cells, the SERS laser assay showed little or no response to the negative control *E. coli*. Likewise, there were no Raman shift peaks observed when all the components of the assay (buffer, antibody, gold-antibody, or cells) were tested individually (Montoya et al. 2003).

Significant effort was expended toward conjugating the anti-Salmonella antibody with colloidal gold in an effort to streamline the procedure so that only a primary antibody need be used. Two different conjugations resulted in similar high-background, non-interpretable results (data not shown), and so this effort was dropped so that a simpler approach without the use of any antibodies could be examined.

A. Salmonella



B. E coli

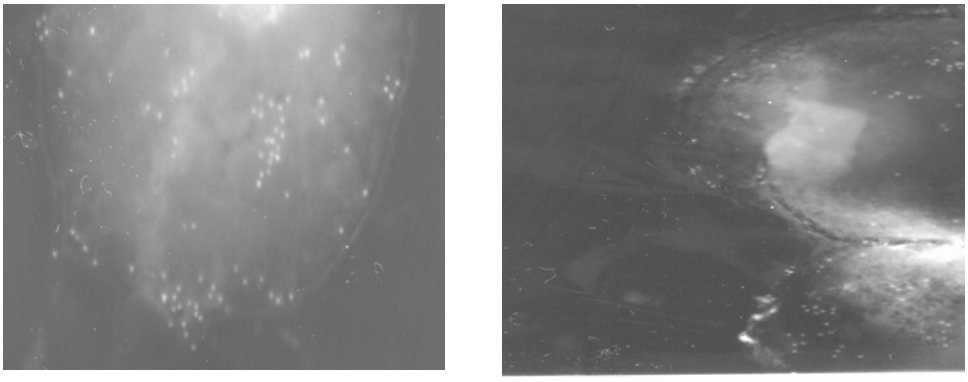


Figure 1. Transmission electron micrographs (50,000 x) of *Salmonella enterica* (A) and *Escherichia coli* (B) after treatment with nano-gold labeled antibodies. The 12-nm size gold colloids are visible as white dots on the 0.5 um x 2 um-sized cells. Though the primary antibody is specific for *Salmonella* and there is heavy binding to the *Salmonella* cells, there is significant binding to the *E. coli* cells.

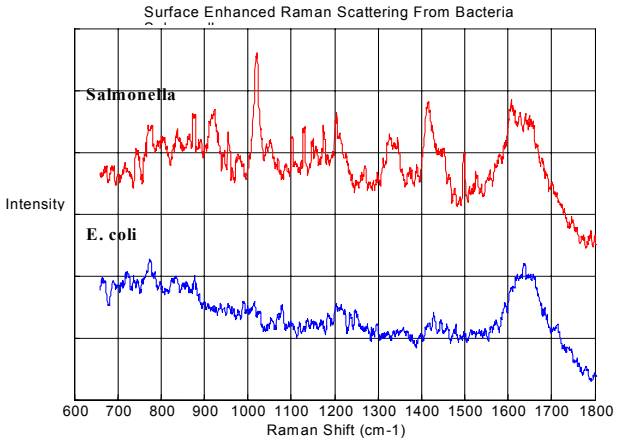
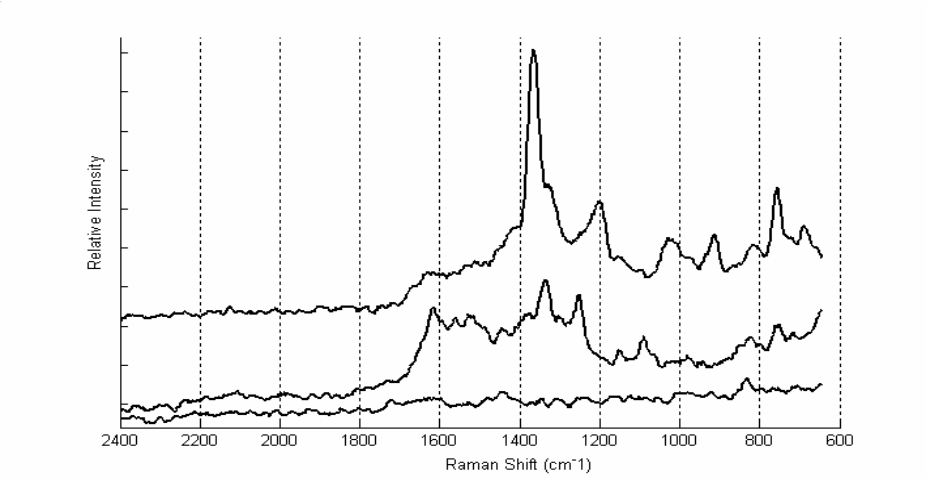


Figure 2**.** Laser irradiation at 532 nm of secondary antibody-gold of the *Salmonella enterica* target and *E. coli* non-target.

Direct SERS Detection Approach

Cells or virus particles were exposed to a SERS-active colloid (silver sols, synthesized with silver nitrate and sodium citrate by R. Montoya, NMSU Physics), aggregated and directly interrogated by laser. In repeated 3-minute assays on separate dates, the following results were consistently observed: the SERS assay was able to distinguish among three species of Gram-positive bacteria (*B. megaterium, B. globigii* strain # 8507, and *B. subtilis* var. niger), but no signals were documented from the Gram-negative *E. coli* (Figure 3A and 3B). Raman shifts from the PP7 virus were typically too complex to interpret due to the high fluorescence (Figure 4). We were able to reduce the fluorescence by changing the laser pump from 532 nm to 632 nm, but no repeatable signals were observed (data not shown).

A.



B.

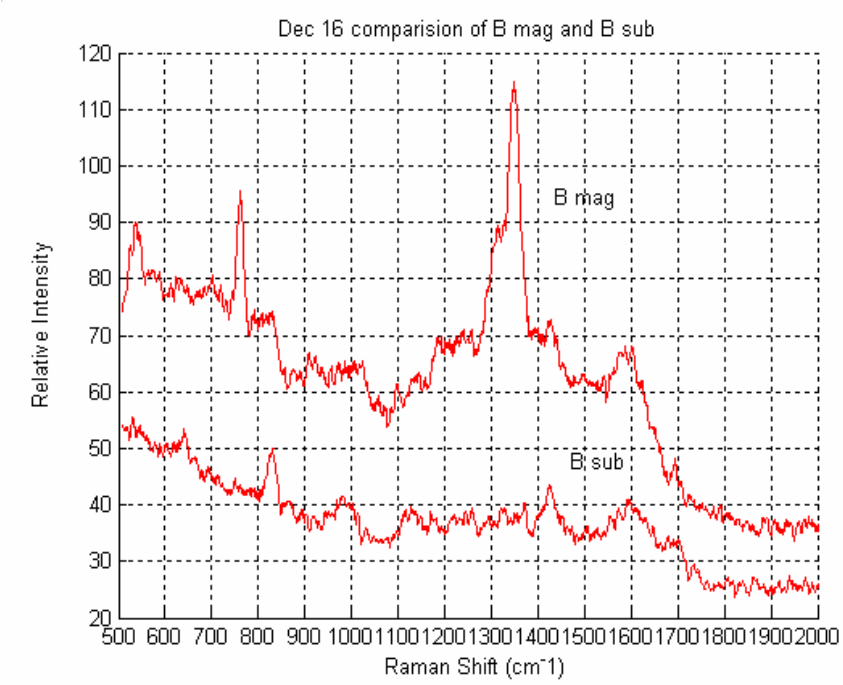


Figure 3. Surface Enhanced Raman Shifts after direct irradiation of bacteria at 532 nm.

A. Three bacterial species exposed to aggregated colloidal silver *(B. megaterium*, Top Trace; *B. globigii*, Middle Trace and *E. coli*, Bottom Trace).

B. Two bacteria exposed to aggregated colloidal silver (B. mag = *B. megaterium*; B. sub = *B. subtilis* var. niger). Note that plot B axis is inverted compared to plot A.

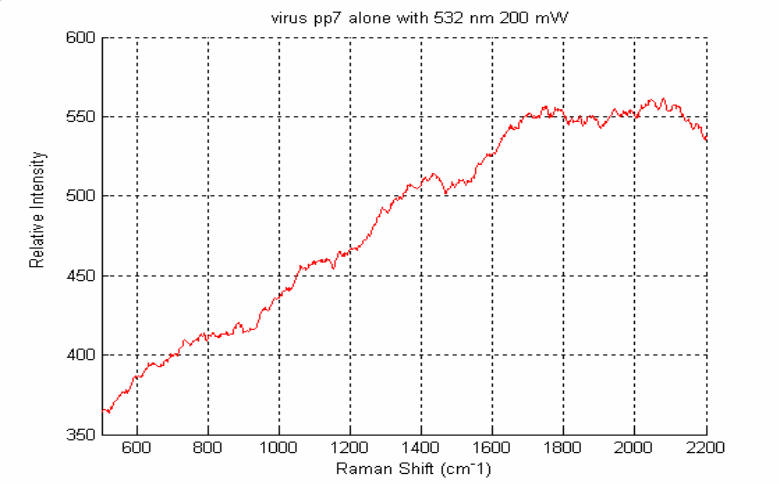


Figure 4**.** Laser irradiation of the PP7 virus at 532 nm. Note the extremely high signal, which shows the potential for resolving a meaningful signal.

SUMMARY

1. Using a dual antibody approach, the SERS-based detection method was able to clearly distinguish between the *Salmonella* target pathogen and non-pathogenic *E. coli* and *Bacillus* species. In contrast, traditional assays (ELISA and electron microscopy) exhibited significant cross-reactivity.

2. A direct SERS-based assay, which took less than 5 minutes to complete, was shown to effectively distinguish between the surrogate for *Bacillu. anthracis* (*B. subtilis* var. niger) and other species of *Bacillus*. Thus, this approach may be useful in complementing other detection schemes to distinguish *B. anthracis* used in bioterrorism from those present naturally. The positive SERS response with Gram- positive but not Gram-negative bacteria is currently hypothesized to be due to the presence of the negatively charged peptidoglycan wall that the laser has access to in Gram-positive but does not have access to in Gram-negative cells due to the presence of the outer membrane. We are currently attempting to exploit the highly complex signal observed with the PP7 virus in order to detect this important group of pathogens.

3. The Raman shifts documented from bacteria in this study correlate well with standards we have run of amino acids, thus appear to be originating from cell surface protein components.

REFERENCES

DePaulo, A. 2002. Detection of Vibrio species in oysters by real-time PCR. American Society for Microbiology, 102'nd General Meeting, Salt Lake City, Utah. May 19-23, 2002.

Fleischmann, M., P.J. Hendra and A.J. Mcquillan. 1974. Ramanspectra of pyridine adsorbed at a silver electrode. *Chem. Phys. Lett*. 26:163.

Kim, W., V.P. Safonov, V.M. Shalaev, and R.L. Armstrong. 1999. Fractals in Microcavities: Giant Coupled Multiplicative Enhancement of Optical Responses. *Rev. Lett.* 82:24.

Kopp, M., A.J. deMello, and A. Manz. 1998. Chemical amplification: continuous-flow PCR on a chip. *Science*. 280:1046-1048.

Montoya, J.R., R.L. Armstrong, G.B. Smith. 2003. Surface enhanced Raman detection of Salmonella antibodies. SPIE Chemical and Biological Sensing IV. 21-25 April21-25, 2003, Orlando, FL.

Ni, J., R.J. Lipert, G.B.Dawson, and M.D. Porter. 1999. Immunosassay readout method using extrinsic Raman labels adsorbed on immunogold colloids. A*nal. Chem*. 71: 4903-4908.

Qi, Y., G.P. Patra, X. Liang, L.E. Willians, S. Rose, R.J. Redkar and V.G. DelVecchio. 2001. Utilization of the *rpo*B gene as a specific chromosomal marker for real-time PCR detection of *Bacilus anthracis*. *Appl. Environ. Microbiol*. 67:3720-3727.

Slot, J.W. and H.J Geuze. 1985. A new method of preparing gold probes for multiple labeling cytochemistry. *Europ. J. Cell Biol*. 38:87-93.

Smith, G.B., J.R. Montoya, C.Y. Esquivias, and R.L. Armstrong. 2004. The use of Raman spectroscopy for the detection of bacterial pathogens and their surrogates. American Society for Microbiology National Meetings. May, 2004, New Orleans, LA.