Sensing and Bio-Sensing Research 8 (2016) 20–26

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

Surface enhanced Raman scattering spectroscopy for detection and identification of microbial pathogens isolated from human serum

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ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form 1 March 2016
Accepted 2 March 2016

Keywords:
SERS
Bacteria
Serum
Diagnostic
Infection
Raman

ABSTRACT

Clinical bacterial diagnostic techniques take between 24 and 48 h and require plating, growth, and examination of colony morphology or color for identification. Biosensors based on Surface Enhanced Raman Scattering (SERS) spectroscopy hold great promise as a platform for rapid and sensitive detection of bacterial pathogens by decreasing time of diagnosis and preventing infection-related morbidity and mortality. The objective of this study was to characterize and evaluate a handheld SERS-based diagnostic system for the detection and identification of bacteria in pooled human sera. Species of *Acenitobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were individually inoculated into pooled human serum samples. Samples were processed by lysis filtration to separate and isolate bacteria. Processed bacterial samples were incubated onto silver nanorod substrates at 60 °C for 3 h. Spectra of bacteria recovered from serum were compared to spectra of pure culture bacteria. Principal Component Analysis and Partial Least Squares Differential Analysis were performed to determine bacterial "molecular fingerprint" (uniqueness and commonalities of measured spectra). Successful detection, identification, and classification of bacteria from human serum using a hand-held Raman spectrometer were demonstrated. Pure culture bacteria were readily identifiable and distinguishable by their SERS-based molecular fingerprints at the species level. Hydrophilic bacteria were readily detected and identified from serum samples without changes occurring to their spectra due to sample processing. Shifts in relative peak intensities of SERS spectra were observed primarily for hydrophobic bacteria after recovery from serum. Bacteria sensitive to lysis filtration require additional reference criteria for SERS identification.

1. Introduction

Bacterial infection is a frequent complication among trauma and surgical patients, both civilian and military. Damage to soft tissues and organs with accompanying immune system suppression enables opportunistic pathogens to colonize and infect [36]. Patients with infection are more likely to be admitted to an ICU, require extensive debridement, have longer lengths of stay in hospital, and have a higher risk of early mortality [16,21,44]. Adverse outcomes within one year after injury with accompanying infection include increased risk of death and repeat hospitalization, greater dependence on healthcare, decreased likelihood to return to work, and diminished overall function [15]. According to the World Health Organization, the prevalence of all healthcare associated infections is 4.5% in the United States, 7.1% in Europe, and 15.5% in underdeveloped countries [2]. The rate of infectious complications in the United States military is approximately 35% for combat casualties [33].

The limitations of current standards of diagnosis can lead to delayed interventions and poor prognosis. Military trauma patients in transit to higher echelons of care present a challenge to healthcare providers due to the disparity in technology available to caregivers outside of the hospital setting [39]. Colonization of combat casualties by infectious pathogens increases during transit through evacuation chains [25] and requires reevaluation at each level of care. Culture-based techniques for standard diagnosis take between 24 and 48 h and require plating, growth, and examination of colony morphology or color to identify bacteria [31]. The exceedingly long turnaround times required for culture-based methods led to the development of DNA- and enzyme-based assays utilizing nucleic acid and antibody probes for identification and quantification of target bacterial cells. Diagnosis using traditional polymerase chain reaction (PCR) requires technical expertise and

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[1] This work was performed while the first author, Dr. Christian N. Kotanen, was working as a Postdoctoral Fellow at the Naval Medical Research Unit San Antonio. Because Dr. Kotanen has already completed his fellowship, the command prefers the corresponding author be a currently employed government representative. None of the listed authors are current government employees; therefore, the Science Director, Dr. John W. Simecek, is being listed as the preferred corresponding author on the manuscript. Please contact Dr. Simecek with questions regarding the corresponding author.

http://dx.doi.org/10.1016/j.sbsr.2016.03.002

sophisticated laboratory equipment. Furthermore, assays with PCR typically require species and/or strain specific probes that may or may not be available for a particular organism. The instability of DNA and antibodies in harsh environments can limit their applicability for use outside of controlled settings.

Advancement in diagnostic capabilities is an important step to improving outcomes of trauma-related infections. Rapid diagnosis, treatment, and control of bacterial infections are necessary to reduce morbidity and mortality. Raman spectroscopy combined with nanotechnology is a potential platform upon which an effective novel diagnostic system can be developed. Devices based on spectroscopic techniques are fast, efficient, and have the potential to reduce the overall time of diagnosis while requiring little to no technical expertise and additional equipment. Raman spectroscopy is a vibrational spectroscopic technique for 'molecular fingerprinting' of tissues, cells, proteins, nucleic acids, and other small organic and inorganic compounds. Raman spectroscopy has been investigated for use in prediction of preterm birth [35] and for the diagnosis of several pre-cancerous and malignant pathologies including basal cell carcinoma, leukemia, prostate cancer, and dysplastic growth in Barrett's esophagus [6,10,30,34].

The addition of nanotechnology further improves the capabilities of Raman spectroscopy. Surface enhanced Raman scattering (SERS) was developed by utilizing nanoparticles as enhancing substrates to improve overall Raman signal intensity, resolution, and limits of detection down to single molecules. Silver nanorod (AgNR) substrates have been developed for detection and identification of viral and bacterial pathogens [11] such as *E. coli* [43], pathogenic mycoplasmas [23], and the human immunodeficiency virus [18]. Enhancement factors of up to 5 x 10^8 have been reported with the length of nanorods directly affecting the measured SERS signal intensity [11,18]. Identification and discrimination of bacteria at the species and strain level using AgNR substrates have been demonstrated [14]. The rapid measurement time, ease of operation, and field deployable potential make this technology a promising alternative infection diagnostic tool for military and civilian caregivers alike.

Benchtop Raman microscopes have been the gold standard for characterization of bacteria with SERS. Benchtop SERS systems are capable of label free detection *in situ* [12], measuring multiple species and strains of bacteria simultaneously [37], discerning between species in mixed bacterial cultures [22], and integrating with microfluidic devices [42]. Raman microscopes are powerful with respect to their resolution and sensitivity, but size and cost restrict their use to the laboratory setting. Raman systems such as the Enwave Raman HRC-10HT [45] and the handheld FirstDefender RM [7] have been developed to address concerns of device footprint and portability. Recently the use of handheld Raman spectrometers for use in SERS identification of bacteria filtered from bean sprout samples have been demonstrated [43]. The objective of this research was to expand the number of bacterial species examined with a field deployable SERS system. The SERS diagnostic system consisting of a handheld Raman spectrometer and multi-well AgNR substrates was characterized with multiple pathogenic bacterial species and strains. Furthermore, the effects of pooled human serum lysis filtration on handheld SERS spectra of pathogenic bacteria were determined in order to characterize the separation of bacteria from increasingly complex biological media.

2. Materials and methods

2.1. Chemicals and reagents

Reagents, common buffers and chromatography grade solvents were purchased from Sigma-Aldrich (St. Louis, MO). UltraPure distilled water (ddH_2O) was purchased from Life Technologies (Grand Island, NY), nutrient agar was purchased from BD Biosciences (San Jose, CA), Tryptic soy broth (TSB) was acquired from BD (Franklin Lakes, NJ), Luria Bertani broth was acquired from Amresco (Solon, OH) and nutrient broth was acquired from Sigma-Aldrich. Filters of 0.45 μm pore diameter were purchased from EMD Millipore (Billerica, MA). Lysis buffer of 0.6% polyoxyethylene-(10)-oleoyl ether in 0.4 M 3-(cyclohexylamino)-1-propane sulfonic acid at pH = 11.7 was prepared followed by filtration through a 0.2 μm filter. Human serum was obtained from Bioreclamation IVT and pooled.

2.2. Silver nanorod substrate preparation

Silver nanorod substrates were acquired from Argent Diagnostics Inc. (Athens, GA). Substrates were prepared by oblique angle deposition technique using an electron beam (e-beam) evaporation system [1,11,43]. Briefly, glass microscope slides were cleaned with piranha solution (80% sulfuric acid and 20% hydrogen peroxide by volume) for 10 min followed by rinsing with deionized water and drying with nitrogen gas. Glass slides were loaded into the e-beam deposition system with the substrate surface perpendicular to the incident vapor direction. A film of 20 nm Ti and followed by 200 nm Ag were evaporated onto glass slides at a rate of 0.2 nm s^{-1} and 0.3 nm s^{-1}, respectively. Substrates were rotated 86° with respect to the incident vapor and silver nanorods were deposited at a rate of 0.3 nm s^{-1}. Thickness was measured using a quartz crystal microbalance. Substrates with nanorod length of 2000 nm were fabricated. A patterning plate mold was applied to the substrate surface and filled with polydimethylsiloxane to form a 4 × 10 array of wells having 3 mm diameter and 1 mm depth.

2.3. Bacterial culture and cell count determination

Bacterial species of *Acinetobacter baumannii* (*A. baumannii*, *ST-3*), *Escherichia coli* (*E. coli*, *ST-11*), *Klebsiella pneumoniae* (*K. pneumoniae*, *ST-9*), *Pseudomonas aeruginosa* (*P. aeruginosa*, *ST-14*) and *Staphylococcus aureus* (*S. aureus*, *ST-19*) were obtained from ATCC and Perkin Elmer (Manassas, VA, and Waltham, MA). Bacterial cells were grown overnight in TSB media at 37 °C in a shaker at 250 rpms. The optical density (OD) of the cells was measured by absorbance spectrometry at 600 nm wavelength. Cultured cells were initially normalized to 1 OD by centrifugation and resuspension in an appropriate volume to bring the concentration to 1 OD/mL. Washing was performed by centrifugation at 14,000 rpms for 5 min, discarding the supernatant and resuspension with ddH_2O. The washing process was repeated three times to remove all broth components followed by a final resuspension of the pellet in ddH_2O back to 1 OD. Cell count was determined by plating the 10^4, 10^3, 10^2, and 10^1 cell dilutions on TSB Nutrient Agar media. Colony forming units (CFU) were counted the following day to confirm bacterial species concentrations of fresh cultures at 1 OD suspended in ddH_2O. Cell concentrations ranged between 10^9–10^{11} CFU/mL at 1 OD. A limit of detection of 10^9 CFU/mL has been reported for the SERS biosensor system under investigation [27]. Fresh pure cultured bacteria suspended in ddH_2O will be referred to as preloaded bacteria.

2.4. Lysis filtration and recovery of bacteria

Lysis filtration is a simple and well established method by which bacteria can be separated from biological matrices such as serum or even whole blood. A lysis filtration method for separation of bacteria from whole blood was adapted from Fothergill et al. [20]. The effects of lysis filtration on bacterial cell concentration and viability have previously been characterized [27]. Preloaded bacteria suspended ddH_2O at 1 OD were first concentrated by an order of magnitude via centrifugation and resuspension in ddH_2O. Concentrated bacteria were pipetted into pooled human serum, 100 μL bacteria into 900 μL serum, and briefly vortexed to generate a bacterial concentration of 1 OD in serum. For purification of bacteria, 500 μL of lysis buffer were added to the serum-bacteria mixture and incubated at room temperature for 2 min. The lysate was then passed through a 0.45 μm filter with vacuum.