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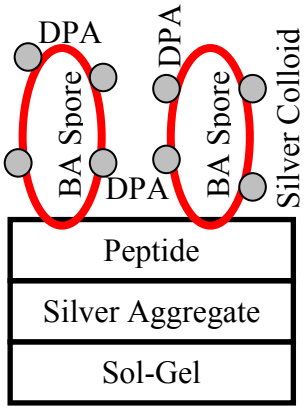
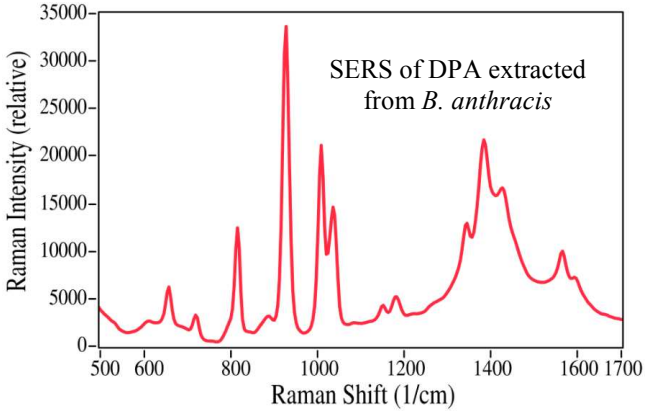
We developed a surface-enhanced Raman spectroscopy (SERS) based assay that allowed selective detection of 1000 *B. anthracis* Ames spores within 15 minutes using dipicolinic acid (DPA) as a biomarker.





Selective detection of 1000 *B. anthracis* spores within 15 minutes using a peptide functionalized SERS assay

Stuart Farquharson*[a], Chetan Shende[a], Wayne Smith[a], Hermes Huang[b], Frank Inscore[a], Atanu Sengupta[c], Jay Sperry[d], Todd Sickler[e], Amber Prugh[e], and Jason Guicheteau[e]

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A surface-enhanced Raman spectroscopy (SERS) assay has been designed to detect *Bacillus anthracis* spores. The assay consists of silver nanoparticles embedded in a porous glass structure that have been functionalized with ATYPLPIR, a peptide developed to discriminately bind *B. anthracis* versus other species of *Bacillus*. Once bound, acetic acid was used to release the biomarker dipicolinic acid from the spores, which was detected by SERS through the addition of silver colloids. This SERS assay was used to selectively bind *B. anthracis* with a 100-fold selectivity versus *B. cereus*, and to detect *B. anthracis* Ames at concentrations of 1000 spores/mL within 15 minutes. The SERS assay measurements provide a basis for the development of systems that can detect spores collected from the air or from water supplies.

Introduction

Since the distribution of *B. anthracis* Ames spores through the US Postal System in 2001, there has been substantial effort to develop technologies that can detect this bioweapon as part of an early warning system. The requirements for a successful technology include the ability to 1) detect as few as 10^4 spores/m^3, the nominal inhalation LD_{50}, for *B. anthracis*,[1,2,3] 2) measure samples in minutes so that evasive action can be taken, 3) perform analysis with sufficient specificity so that commonly occurring *Bacilli* do not produce false-positive responses, and 4) operate where the attack occurs. The most successful technologies developed to detect *B. anthracis* spores since the 2001 attack include polymerase chain reaction (PCR) systems and immunoassay kits. However, neither satisfies all of these requirements.[4] While PCR provides exceptional specificity, measurements still require an hour or more and portability is limited.[5,6] In contrast, immunoassays are very portable, but are sensitive to environmental temperature and lack sufficient specificity,[7,8] due to the fact that the surface antigens of other *Bacilli* that are common to the environment, such as *B. cereus*, bind to the antibodies used in the assays.

Recognizing the limitations of these and other technologies being developed,[9-23] we have been investigating the ability of surface-enhanced Raman spectroscopy (SERS) to meet the listed requirements for successful detection and identification of *B. anthracis* spores. The approach is based on 1) the detection of dipicolinic acid (DPA) as a biomarker,[24] since it represents ~10% of the spore weight in the form of calcium dipicolinate (CaDPA);[25,26] 2) the measurement of 10 ng/mL DPA by

SERS;[27,28] 3) the extraction of DPA from spores;[29,30] and 4) the portability of current Raman spectrometers. However, previous SERS measurements of *Bacillus* spores have lacked the required sensitivity and selectivity. Specifically, SERS has been used to detect DPA extracted from ~10^7 spores/mL *B. cereus* and *B. subtilis* using hot dodecylamine and nitric acid combined with sonication, respectively.[31,32] While the unique SER spectrum of DPA limits false positive responses to CaDPA-containing bacteria, primarily *Bacillus* and *Clostridium*, additional discrimination is needed to eliminate false positives due to other *Bacillus* species.

To achieve selectivity at the species level, we recently functionalized silver nanoparticles with peptides, and demonstrated discriminate SERS detection of *B. anthracis* against both *B. cereus* and *B. subtilis*.[33] Unfortunately the addition of the peptide likely dampened the plasmon field responsible for the SER effect,[34] as only 10^9 spores/mL could be detected. Here we describe the addition of a second SER-active material to the assay in the form of a silver colloid that improved sensitivity by ~6 orders-of-magnitude over our recent SERS measurements, while at the same time achieving an ~100-fold selective binding of *B. anthracis* versus *B. cereus*.[33] The SERS assay was developed using the avirulent *B. anthracis* Sterne strain, which is missing the $pXO2^-$ plasmid necessary to produce the anthrax toxins.[35] The assay was then used to measure 1000 spores/mL of the virulent *B. anthracis* Ames strain within 15 minutes and 100 spores/mL within 25 minutes.

Experimental

All chemicals, reagents, and solvents, including those used to prepare the SER-active sol-gels, were purchased and used as received from Sigma-Aldrich (Milwaukee, WI). *B. cereus* and *B. anthracis* Sterne samples were obtained from American Type Culture Collection (Manassas, VA), and prepared at the University of Rhode Island (URI). Samples, beginning with 1 mL wet suspensions of *B. cereus* and *B. anthracis* Sterne spores, were diluted by a factor of 300, such that direct microscope counts could be performed. *B. anthracis* Ames samples were prepared at the US Army's Edgewood Chemical Biological Center (ECBC) in a Biosafety Level 3 (BSL3) laboratory. ECBC concentrations were verified by serial dilution of the spores in water, plating on trypticase soy agar, incubating for 24 hours, and counting for viability. All samples were then diluted to produce the measured concentrations. Peptides of the sequence ATYPLPIR were custom synthesized by New England Peptide (Gardner, MA) following standard solid-phase 9-fluorenylmethyloxycarbonyl chemistry. Glass capillaries, Tygon tubing, syringes and syringe ports were obtained from VWR Scientific (Arlington Heights, IL). Acrylic plates were machined to hold the SERS-active capillaries.

The silver-doped sol-gels were prepared according to published procedures by mixing a silver amine precursor and an alkoxide precursor at 1:1 v/v.[31] The silver amine precursor consisted of a 1:1:2 v/v/v ratio of 1N $AgNO_3$/28% NH_4OH/CH_3OH, while the alkoxide precursor consisted of methyltrimethoxysilane. The SERS capillaries were prepared by drawing 20 µL of the silver-doped sol-gels into 10 cm long, 0.8 mm inner diameter glass capillaries to produce ~1 cm long segments. The segments were allowed to gel and cure for 12 hours, then the incorporated silver ions were reduced with dilute $NaBH_4$. Silver colloids were prepared from $AgNO_3$ and $NaBH_4$ according to literature and used within 1 hour of preparation.[36] The reduced silver in the sol-gel produced fused aggregates of particles with diameters in

the range of 50 to 150 nm, while the reduced silver colloids produced particles with diameters in the range of 100 to 400 nm (see Electronic Supplementary Information,† Fig. ESI1). The silver-doped sol-gels and silver colloids allowed detecting 1 µg/mL dipicolinic acid in water with large signal to noise ratios (S/N) (Fig. ESI2).

The peptide functionalization of silver nanoparticles was carried out by adding 1 to 50 µg/mL peptide solution to the SER-active sol-gel segments immobilized within the glass capillaries. The peptide was allowed to bind for 30 minutes, and then water was passed through the capillary to remove any unbound peptide. Monolayer coverage was determined to be ~10 µg/mL peptide by adding 100 µg/mL DPA to the silver-doped sol-gels, which were functionalized with increasing amounts of peptide, until no SERS of DPA was observed (Fig. ESI3).

B. cereus and *B. anthracis* Sterne samples were measured in a BSL2 hood and laboratory at URI. *B. anthracis* Ames samples were measured in a BSL3 cabinet and laboratory at ECBC. An acrylic plate, containing SER-active capillaries with 30 cm of tubing connected to each end of each capillary, was placed on an XY positioning stage (Conix Research, Springfield, OR). All of the exit tubing was draped into a container of bleach. The *B. anthracis* Ames samples at 10^4, 10^3, or 10^2 spores/mL, were drawn, 20 mL each, into 60 mL syringes. The syringes were mounted on a dual-syringe pump (Kent Scientific, Torrington, CT) and connected to the tubing attached to the capillaries using syringe fittings.

For SERS measurements the capillaries or acrylic plates were mounted on the XY stage, such that the focal point of an f/0.7 aspheric lens of a fiber optic probe was just inside the capillary glass wall. The plates included a capillary containing cyclohexane that served as an x- and y-axis Raman spectral reference, as well as a stage positioning reference. A software program was used to measure spectra at 10 points spaced 1 mm apart along the length of the metal-doped sol-gel segment. Preliminary measurements using *B. cereus* and *B. anthracis* Sterne were performed using a Fourier transform Raman spectrometer (Real-Time Analyzers), equipped with a 785 nm diode laser (Innovative Photonic Solutions, Monmouth Junction, NJ) and a Si-photo-avalanche detector (Perkin Elmer, Stamford, CT). The system was used to deliver 75 mW of power and collect 1 minute spectra at each position at 8 cm^{-1} resolution. For measurements of *B. anthracis* Ames at ECBC, a dispersive Raman spectrometer (Real-Time Analyzers) equipped with a CCD (Andor, UK) was used to collect a 200 to 2000 cm^{-1} spectral range with an average resolution of 16 cm^{-1}. For these measurements the fiber optic probe was remotely connected via 10 meter fiber optic cables to the laser source and Raman spectrometer outside the BSL3 laboratory. A 785 nm diode laser (Innovative Photonic Solutions, NJ) was used to deliver 75 mW of power at the sample and collect 10 sec spectra at each position. The XY stage was connected via a 10 m serial cable to the Raman computer, also outside the BSL3 laboratory.

Results and Discussion

The design of the *B. anthracis* assay consists of silver nanoparticles functionalized with peptides distributed throughout a porous glass structure so that sample and reagents can easily flow through (Fig. 1). Peptides were chosen instead of antibodies for their tolerance to heat and moisture, which extends their usable lifetime and makes them more suitable for field use. For this specific application,

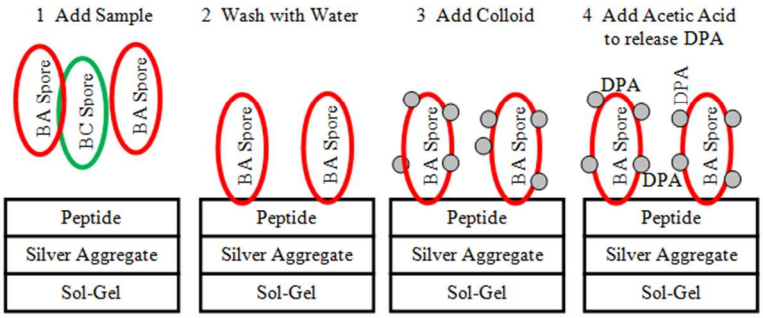

Fig. 1. Illustration of the assay steps used to detect *B. anthracis* (BA). 1) A liquid sample is drawn into the capillary so that any *B. anthracis* spores that might be present bind to the peptides. 2) Water is drawn through the capillary to remove any unbound chemicals and biologicals that might interfere with the measurement. 3) Silver colloid is drawn into the capillary to provide a SER-active material. 4) Acetic acid is drawn into the capillary to cause the release of DPA. 5) The capillary is placed in a Raman spectrometer and the SERS of DPA is measured (not shown). Peptide used: ATYPLPIR-C.

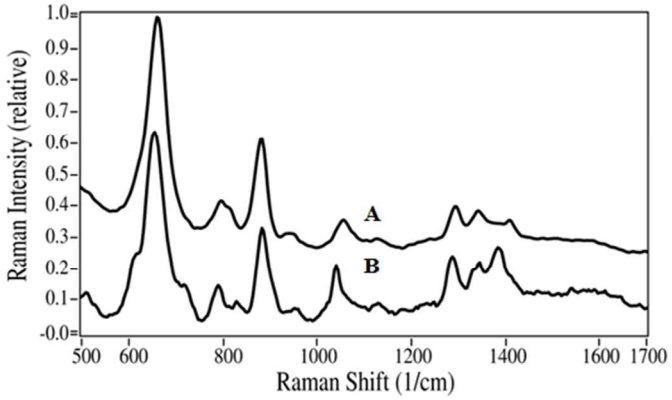

Fig. 2. SERS of A) 1 mg/mL ATYPLPIRC peptide functionalized on silver particles within the sol-gel and B) 1 mg/mL cysteine on silver particles within the sol-gel. Spectral conditions: 75 mW of 785 nm laser excitation, ten 1 min spectra averaged using a Fourier transform Raman spectrometer. Spectra are displayed on same intensity scale, but offset for clarity.

the ATYPLPIR peptide was selected for its essentially identical concentration dependent binding of *B. anthracis* Ames and Sterne with substantial discrimination against *B. cereus* for $5x10^8$ spores/mL samples as measured by fluorescence-activated cell sorting.[37,38]

The first step in developing the assay involved incorporating a 1 cm long porous glass plug within a 1 mm diameter glass capillary that contained silver nanoparticles distributed throughout. This was accomplished by mixing a silver amine with a silica-based alkoxide to produce a metal-doped sol-gel that could be drawn into the capillary and cured. The second step involved functionalizing the silver particles with the peptide. This was accomplished by attaching a cysteine residue to the C-terminus of the peptide sequence, viz. ATYPLPIR-C, such that the cysteine sulfur could bind to the silver surface.[33] The amount of peptide, 50 µg/mL, was selected to produce at least a monolayer on the silver surface and maximize the opportunity for spore binding, while minimizing potential interfering spectra from other chemicals and biologicals in the sample. Successful functionalization was determined by

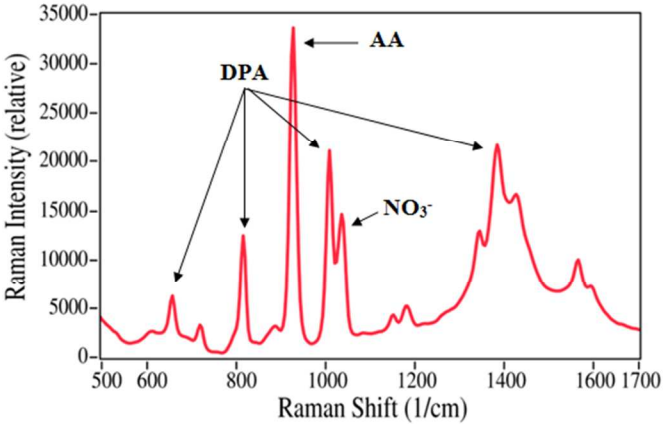

Fig. 3. SERS of *Bacillus anthracis* Sterne at 10^4 spores/mL. Sample conditions: 10 µL sample, 10 min incubation, 1 mL of water wash, 0.2 mL silver colloid addition, and 0.2 mL acetic acid (AA) addition. Spectral conditions: 75 mW of 785 nm laser excitation, ten 10 sec spectra, 1 mm apart along capillary length, averaged using a dispersive Raman spectrometer.

measuring the SERS of the peptide bound to the silver. The spectrum is virtually identical to that of cysteine, both dominated by an intense sulfur-carbon vibrational mode at 657 cm^{-1} (Fig. 2).[39] The other amino acids of the peptide do not contribute significantly to the spectrum.

The assay was then optimized in terms of sample and reagent volumes and incubation (binding) time by measuring the avirulent *B. anthracis* Sterne strain at URI. For these measurements, an ~10 µL sample was injected into the peptide functionalized, silver-doped, sol-gel capillaries and allowed to incubate for 10 minutes. 1 mL of water was drawn through the capillaries to remove unbound spores. Then 0.2 mL of freshly prepared silver colloid was added to the sol-gel capillary to promote SERS, followed by 0.2 mL of acetic acid to cause the release of DPA from the spores.

The order of the reagent addition is important, in that adding the colloid first allows the particles to disperse throughout the sol-gel containing the peptide captured spores, while the acetic acid, in addition to causing the release of DPA, causes the particles to aggregate increasing the plasmon field strength.[40] Acetic acid, a known biocide,[41] was investigated as a replacement for dodecylamine and nitric acid, which required heat and sonication, respectively, to effect DPA release.[28,29] It was found that acetic acid caused release of DPA in a few minutes at room temperature reducing the requirements for field use. It is not known if acetic acid promotes germination or degrades the spore wall as suggested for dodecylamine and strong acids, respectively.[42,43] In either case, acetic acid is ideal for this application, as it causes immediate release of DPA and silver particle aggregation.

SER spectra with a high S/N were obtained for 10^4 spores/mL and higher concentrations (Fig. 3). The spectra are very similar to the normal Raman spectra of DPA in solution with only minor peak shifts, suggesting a modest interaction between the carboxylic acid groups and the electropositive silver particles.[28] The SER spectrum is dominated by DPA peaks at 659, 818, 1007, 1381, and 1567 cm^{-1}, which have been assigned to a CC ring bend, a CH out-of-plane bend, the symmetric pyridine ring stretch, an OCO symmetric stretch, and an OCO asymmetric stretch, respectively.[31] The spectra also contain a peak at 930 cm^{-1} due to acetic acid, and a

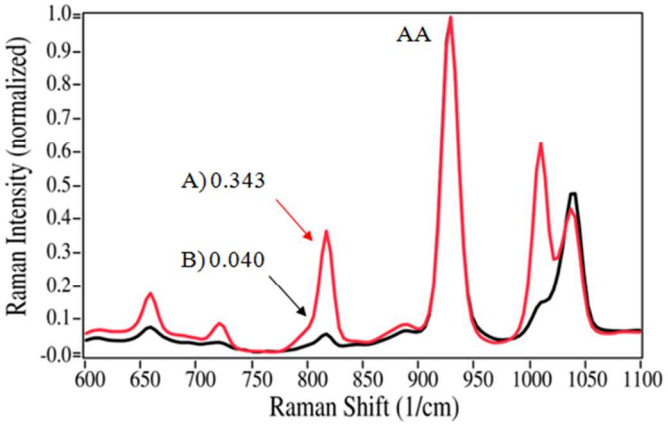

Fig. 4. SERS of A) 10^4 *B. anthracis* Sterne spores/mL and B) 10^5 *B. cereus* spores/mL on a *B. anthracis* Sterne assay. All conditions as in Fig. 2, except spectra are the average of three capillaries and normalized to the acetic acid peak height at 930 cm^{-1} and baseline at 775 cm^{-1}. Listed peak heights are baseline corrected.

peak at 1044 cm^{-1} due to nitrate (NO_3^-). Ten points along the sol-gel plug within the capillary were measured and averaged to ensure that a spectrum representative of the sample was obtained. The detection of 10^4 spores/mL represents ~5 orders-of-magnitude improvement over previous measurements that used a nearly identical procedure, except the silver colloid was not added. This clearly suggests that most of the SERS is generated by the colloid. It is worth noting that the total time to perform these measurements was ~13 minutes: 10 min for binding, ~1 min total for sample and reagent additions, and 1.67 min to collect the 10 spectra at 10 sec each.

Specificity of the assay was demonstrated by measuring *B. cereus* spores following the same procedure using identically prepared peptide-functionalized silver-doped sol-gels. *B. cereus* was chosen as it is genetically one of the most closely related *Bacilli* to *B. anthracis*.8 For a 10^4 spore/mL *B. cereus* sample, no SERS of DPA was obtained, so a 10^5 spores/mL sample was measured. This higher concentration produced a weak DPA spectrum, suggesting that a degree of non-specific binding to the peptide does occur. The amount of non-specific binding was estimated by comparing the 818 cm^{-1} peak height for the two samples normalized to the intensity of the 930 cm^{-1} acetic acid peak, which, at constant concentration, was used as an internal intensity reference. In an effort to quantify specificity with a degree of reproducibility, three capillaries were measured and the spectra averaged for each spore type. The averaged 818 cm^{-1} peak height for 10^5 spore/mL *B. cereus* was ~12% of the averaged 818 cm^{-1} peak height for 10^4 spores/mL *B. anthracis* Sterne, suggesting ~2 orders-of-magnitude selectivity in favor of *B. anthracis* Sterne (Fig 4.).

The assay procedure developed for *B. anthracis* Sterne with the same peptide was used for the measurements of *B. anthracis* Ames at ECBC, but the sample and reagent volumes were modified. The reagent volumes were increased to 0.5 mL to compensate for the additional syringe-to-capillary tubing required by the BSL3 laboratory setup. The sample volume was increased to 5 mL and delivered using a syringe pump at 1 mL/min to minimize variability associated with manual sample introduction to the assay. While the increase in sample volume effectively increased the number of spores introduced to the assay, it also decreased the incubation time to

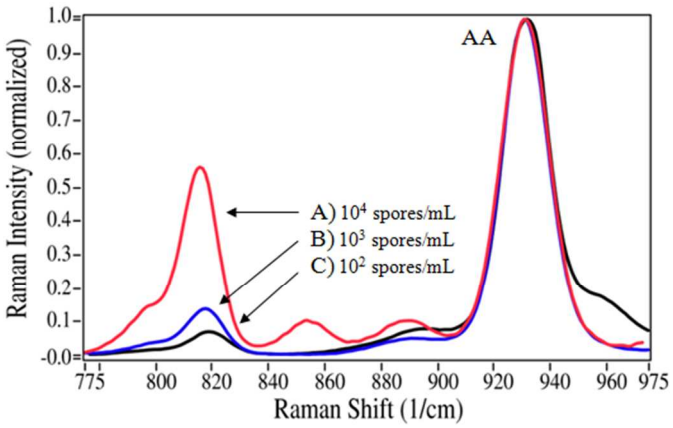

Fig. 5. SERS of *B. anthracis* Ames at A) 10^4, B) 10^3, and C) 10^2 spores/mL; normalized to AA peak intensity and baseline offset. All conditions as in Fig. 3, except 5 or 10 mL sample (see text) flowed at 1 mL/min and 0.5 mL reagents, and spectra are normalized as in Fig. 4.

5 minutes. Quality spectra were obtained for samples of *B. anthracis* Ames at both 10^3 and 10^4 spores/mL in ~13 minutes (5 min to load the sample, 2 min each to load the water, colloid, and acetic acid, and 1.67 min for the SERS measurement, Fig. 5).

Based on these successful measurements, a 10 mL sample of 10^2 spores/mL *B. anthracis* Ames was measured. In addition to a larger sample volume, a 5 minute incubation period was added to ensure a reasonable S/N. The 818 cm^{-1} peak height for 10^2 spores/mL sample is ~60% of the 10^3 spores/mL sample (Fig. 5), which is reasonable given that the former sample volume was doubled and a 5 minute incubation time was added compared to the latter sample.

Comparison of the flow to the static measurement sensitivity based on DPA SERS peak heights indicate that a significant amount of the spores pass through the sol-gel during flow. Without knowledge of the yet unknown binding constant for this peptide-spore combination, it is not possible to determine if the spores simply pass through, are displaced, or released.

Conclusions

This study successfully demonstrated the combined ability of silver colloids with peptide-functionalized silver particles to detect 10^3 *B. anthracis* Ames spores/mL within 15 minutes with ~2 orders-of-magnitude selectivity versus other *Bacilli*. The sensitivity of this SERS assay suggests that an early warning system could be developed capable of detecting the required 10^4 *B. anthracis* spores/m^3 in ~10 minutes. Consider that a typical aerosol collector, capable of extracting particles from ~5 m^3 air into a 5 mL sample reservoir every 10 minutes,[44] could provide the SERS assay with 10^4 spores/mL every 10 minutes. This concentration is a factor of 10 greater than the 10^3 spores/mL presented here. It is also worth noting that similar peptide-based SERS assays could be developed for food and waterborne pathogens, provided that the selective peptides and appropriate biomarkers are available.

Acknowledgements

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[a] *Real-Time Analyzers, Inc., 362 Industrial Park Road, Unit 8, Middletown, CT 06457, USA.*
[b] *Smiths Detection, 14 Commerce Dr, Danbury, CT 06810, USA*
[c] *Dr. Reddy's Laboratories, Bachupally 500090, Andhra Pradesh, India*
[d] *Department of Cell and Molecular Biology, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881*
[e] *US Army Edgewood Chemical Biological Center, 5183 Blackhawk Road, Aberdeen Proving Ground, MD 21010, USA*
[*] *Real-Time Analyzers, Inc., E-mail: stu@rta.biz; Fax:860-635-9804; Tel: 860-635-9800*
† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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