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A Portable Immunoassay Platform for Multiplexed Detection of Biotoxins in Clinical and Environmental Samples

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C.-Y. Koh,[a] M. E. Piccini,[a,b] U. Y. Schaff,[a,c] L. H. Stanker,[d] L. W. Cheng,[d] E. Ravichandran,[e] B.-R. Singh,[e] G. J. Sommer,[a,c] and A. K. Singh[a] ,

Multiple cases of attempted bioterrorism events using biotoxins have highlighted the urgent need for tools capable of rapid screening of suspect samples in the field (e.g., mailroom and public events). We present a portable microfluidic device capable of analyzing environmental (e.g., white powder), food (e.g., milk) and clinical (e.g., blood) samples for multiplexed detection of biotoxins. The device is rapid (<15-30 min sample-to-answer), sensitive (< 0.08 pg/mL detection limit for botulinum toxin), multiplexed (up to 64 parallel assays) and capable of analyzing small volume samples (< 20 µL total sample input). The immunoassay approach (SpinDx) is based on binding of toxins in a sample to antibody-laden capture particles followed by sedimentation of particles through a density-media in a microfluidic disk and quantification using a laser-induced fluorescence detector. A direct, blinded comparison with a gold standard ELISA revealed a 5-fold more sensitive detection limit for botulinum toxin while requiring 250-fold less sample volume and a 30 minute assay time with a near unity correlation. A key advantage of the technique is its compatibility with a variety of sample matrices with no additional sample preparation required. Ultrasensitive quantification has been demonstrated from direct analysis of multiple clinical, environmental and food samples, including white powder, whole blood, saliva, salad dressing, whole milk, peanut butter, half and half, honey, and canned meat. We believe that this device can met an urgent need in screening both potentially exposed people as well as suspicious samples in mail-rooms, airports, public sporting venues and emergency rooms. The general-purpose immunodiagnostics device can also find applications in screening of infectious and systemic diseases or serve as a lab device for conducting rapid immunoassays.

Introduction

Biotoxin attacks in the last decade ([1]Ricin letters, Apr 2013) have highlighted that biotoxins remain a credible bioterrorism threat. What makes these toxins attractive to bad actors (both individual and state) is that they are one of the most toxic substances known to man and hence, minute amounts are sufficient to kill large number of people rapidly[2-4]. One gram of botulinum toxin, evenly dispersed, has the potential to incapacitate 100,000 adults[2] and anthrax toxin has a mortality rate of 85% without treatment[3]. Some biotoxins, such as ricin, may not cause significant mortality but can cause widespread panic and potentially high morbidity. The socio-political and economical costs are too high for biotoxin attacks to have delayed, missed or inaccurate detection even in cases where the attack may not lead to many deaths. Even low-scale biotoxin letter attacks can shut down large swaths of mail system and buildings. A successful widespread dispersal of a toxin would be devastating to society; it is estimated that botulinum exposure to 100,000 people would result in 30,000 deaths and a total economic cost of $8.9 billion due to costly and long-term management of intoxication[5]. Advances in biotechnology have greatly lowered the barrier in terms of equipment and training needed for individuals to access, develop, and produce lethal amounts of toxins.

Because of the severe morbidity and mortality that biotoxins can cause, early detection is paramount for effective deployment of both medical and non-medical countermeasures. Rapid detection will not only lead to faster treatment (potentially saving lives) but also in containment of the attack by identification and elimination of the source(s). In events involving mail or other small-scale attacks, we need deployable devices for rapid, point-of-incident, high-confidence detection of multiple biotoxins. In case of large attacks, the need for rapid, cost-effective detection and diagnostics is paramount where early detection will not only limit the extent of exposure and identify the food or other source of toxin quickly, but also assist with effective rationing of anti-toxin supplies, reduce patient loads in primary care facilities, and assuage the large numbers of "worried-well" seeking treatment[6]. Early detection

is equally important in natural cases of toxin exposure (e.g., shiga, botulinum) to accurately guide the therapy and identify the contaminated food source. For botulinum neurotoxins, antitoxin therapy is most effective if it is administered while toxin remains free in the circulation[7]. The onset of symptoms can be within hours after exposure (average 18 to 36 hours with ingestion), and once toxin has impaired nerve function, victims frequently require mechanical ventilation and intensive care for 6-8 weeks at a cost of $250,000 - $500,000 while toxin is cleared from intoxicated neurons[4]. Ricin intoxication can lead to gastrointestinal hemorrhage after ingestion, and acute pulmonary disease upon inhalation[8]. Ingested ricin is absorbed within the first 2 hours by lymphatic and blood vessels and accumulates in the liver and spleen, while inhaled ricin quickly damages lung tissue. With inhalation exposure to ricin, signs of intoxication may not appear for 12 to 24 hours, but microscopic damage to lung tissue may occur by 8-12 hours and irreversible biochemical changes may occur by 60 to 90 minutes post-exposure making therapy difficult[9]. Staphylococcal enterotoxin B (SEB) produces a syndrome of fever, nausea, and diarrhea and may produce a pulmonary syndrome if aerosolized. Upon inhalation, SEB is transferred into circulation within 30 to 90 minutes and toxicity is observed as early as 4 hours post-exposure[6]. According to the Centers for Disease Control, treatment for ricin, shiga, and SEB are limited to supportive care, with aggressive administration of fluids, pain management aids, and hospitalization. For anthrax infections, administration of antibiotics is suggested with continuous fluid drainage and mechanical ventilation. In the case of contact with spores, antitoxins are administered to decrease the likelihood of spore germination.

Despite the urgent need, no rapid, point-of-care and point-of-incident detectors or assays exist to provide high-sensitivity detection of biotoxins. The standard methods for detection are laboratory tests (typically ELISAs or mouse bioassays) – one test for each toxin, requiring long turn-around time, specialized labs, and specific expertise. For example, live-mouse bioassay is the only FDA-approved metric for confirming the presence of active botulinum toxin in a sample[7, 10-12]. The mouse bioassay, while sensitive, requires days for result confirmation thus rendering it ineffective for timely therapeutic mitigation. The mouse assay is also extremely costly and public health laboratories spend millions of dollars every year to maintain and operate a mouse facility for screening. Many sandwich immunoassays have been reported for biological toxins but these are slow, involve multiple steps and require specialized laboratories, and many are not amenable to complex sample matrices such as serum or food[13-16]. For example, SEB, which may be detected in blood, urine, or respiratory secretion, is typically detected by a microtiter plate-based ELISA performed by a trained person in a laboratory and takes hours to complete. In a suspected bioterrorism incident samples are collected, preserved and shipped to specialized laboratories for analysis, wasting precious time before a positive identification is made. There are a few fieldable assays (strip or lateral-flow immunoassays) available [17, 18] but they have major limitations—1) provide only qualitative results, 2) detect one toxin only per assay and 2) have unacceptable specificity and sensitivity thereby necessitating a second confirmatory test that may take 2-4 days for confirmation.

To meet the urgent need for field-portable, multiplexed biotoxin detection systems, we have developed a simple, sample-to-answer microfluidic platform that vastly outperforms conventional ELISA while has the speed typical of a lateral-flow assay. It employs a sedimentation-based centrifugal immunoassay and requires no sample preparation for clinical, food, and environmental samples. This is exemplified in the work with agents of concern for biodefense: specifically a panel of biotoxins including botulinum, ricin, anthrax toxin protective antigen (PA), botulinum neurotoxins A and B, SEB, and shiga toxin. We further demonstrate quantification of toxins without any loss of sensitivity directly from untreated food samples including canned meat, carrot juice, peanut butter, and salad dressing with no additional sample preparation.

Materials and Methods

SpinDx development

The SpinDx prototype device utilizes an epifluorescent optical system. A 635 nm diode laser (4 mW, Edmund Optics) passes through a custom-sized band pass excitation filter (640 nm, 14 nm BW, Semrock) and a 676 nm, 29 nm bandwidth emission filter (Semrock). The other physical components of the optical hardware (cages, holders) were purchased from Edmund Optics and Thor Labs. A photomultiplier tube (Hamamatsu) was used for detection of the signal. A custom stepper motor (Lin Engineering) was used to rotate the disk and provide positional accuracy for channel registration. Electrical components were purchased from Mouser. System control was provided by a Stellaris series motherboard (Texas Instruments). Other electrical connections were facilitated by custom-designed printed circuits boards (fabricated by ExpressPCB). Communication to the device was achieved via USB or Bluetooth connections. Software was created in-house using Code Composer (Texas Instruments). Labview was used to create the GUI. The device housing was designed in-house and fabricated by ProtoMold.

Reagent preparation

A general scheme for antibody conjugation to the microparticles is shown in Supplemental Figure S1. Conjugation of the capture antibody to the microparticle proceeded via standard carbodiimide chemistry. 10 mg of silica microparticles pre-functionalized with carboxylic acid groups were activated with an excess of EDC and NHS (0.5 mmoles of EDC, 0.5 mmoles of NHS) at pH 6.4 in 1 mL of 100 mM MOPS to form the succinimidyl ester. The particles were washed first with MOPS and subsequently with PBS. The capture antibody was added to a concentration of 4 μM and the solution was raised to pH 8.15 with 1M NaHCO3 and reacted at 4 °C for two hours. Any remaining activated ester was quenched with 200 mM glycine and washed in PBS three times. The particles were then twice blocked with 1% BSA for 30 minutes at 4°C. The particles were then washed in wash buffer (0.05% (w/v) Tween-20, 0.05% (w/v) Pluronic F127, 0.05% (w/v) n-dodecyl β-D-maltoside, 0.76 mM NaN3, 0.1% (w/v) BSA, in PBS) and resuspended in wash buffer to a concentration of 5% solids. The surface of the PMMA microparticles were first functionalized with PEG bis(amine) through transacylation of the methyl ester groups at pH 12.5 in 235 mM sodium borate. 250 μL of a 10% solids suspension of PMMA microparticles were diluted to 0.5% solids in borate buffer. 50 mg of PEG bis(amine) was added and allowed to react overnight at room temperature with mixing. The microparticles were washed with PBS. Determination of functionalization was determined by conjugation to FITC in

500 mM carbonate buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$; pH 9.5) followed by fluorescence spectroscopy with excitation at 488 nm and emission at 520 nm. Surface amines were then converted to carboxylic acids by the addition of 25 mg of succinic anhydride in DMF with triethylamine (1 mg/mL). The particles were then washed extensively in PBS and resuspended in MOPS. The carboxylic acids were converted to succinimidyl esters with EDC/NHS and capture antibodies were conjugated as above. The surface of polystyrene microparticles was functionalized through electrophilic aromatic substitution. Polystyrene microparticles were suspended in DCM with 50 mg of Fmoc-NH-PEG acid chloride (converted from the carboxylic acid with neat oxalyl chloride and used without purification) and catalytic aluminum trichloride. The reaction proceeded for 2h at 30 °C with mixing. The particles were then washed in methanol followed by water. The Fmoc group was removed with 5% aqueous ammonia. The amine was converted to a carboxylic acid with succinic anhydride. The resultant carboxylic acid was converted to a succinimidyl ester and conjugated to the capture antibody as above.

Oligonucleotide-microparticle conjugation

PMMA microparticles with carboxylic acid functionalized surfaces were prepared as above. Surface carboxyls were activated with EDC/NHS and covalently bonded to the substrate via the amine on the 5' end of the stem-loop substrate.

Antibody-quantum dot conjugation

Detection antibodies were labeled with quantum dots using standard EDC/NHS carbodiimide chemistry. Carboxylic acid-terminated quantum dots were diluted in 100 mM sodium borate to a concentration of 600 nM. Detection antibodies added to this mixture to a final concentration of 500 nM. To this mixture 1 nmol of EDC/NHS was added and the reaction proceeded at room temperature for 30 minutes with gentle stirring. The reaction was spun through a desalting column made of Sephacryl S400HR and the first fraction was taken as the quantum dot-antibody conjugate. Degree of labeling was determined using the published value for UV absorption of the quantum dot and the protein concentration was determined using BCA.The labeled antibody was stored in PBS with 1% wt/vol BSA as a carrier.

SpinDx immunoassay protocols

Immunoassays were performed in triplicate. Standard curves were collected in 50% FBS as the sample matrix. Shiga-like toxin, ricin, or SEB were serially diluted in FBS. To 7 µL of a 5% solids suspension of capture particles was added 1 µL of a 300 nM solution of quantum dot-labeled detection antibody. To this suspension was added 7 µL of the sample to yield 20 nM final concentration of detection antibody. The suspension was incubated with mixing for 20 minutes at room temperature. Each channel of the disk was preloaded with 3 µL of a density medium consisting of 90% Percoll in PBS with 0.05% Tween 20, 0.1% BSA, and 0.1% F127. After incubation, 4 µL of the suspension was added to the channel and the disk was spun at 8000 rpm for 45 s. The bead pellet was analyzed on an Olympus IX-70 fluorescence microscope (Center Valley, PA) with 405 nm excitation and 705 nm emission, a CoolSnap HQ interline CCD camera (Roper Scientific, Trenton, NJ) and Image-Pro Plus imaging software (MediaCybernetics, Bethesda, MD). The average fluorescence of each bead pellet was measured and compared with calibration curves generated in parallel with standard dilutions to quantify the target analyte. For food matrices, some sample treatment was performed: spinach and ground beef were homogenized with PBS in a mortar and pestle and then used without further treatment as above.

Toxin activity assay protocols

For ricin and shiga-like toxin, toxins were incubated with microparticle-conjugated stem-loop substrates for 20 or 240 min in 10 mM potassium citrate at pH 4.0. To this suspension, labeled APE1 was added to a final concentration of 100 nM and PBS (pH 7.4) with 50 mM EDTA and allowed to react for 20 min. After incubation, 4 µL of the suspension was added to the channel and the disk was spun at 8000 rpm for 45 s. The bead pellet was imaged on the same optical equipment as above with 405 nm excitation and 585 nm emission. For SEB, MHC II was immobilized to 1 µm carboxylic acid functionalized microparticles. Jurkat cells were fixed in ice cold methanol for 5 min, washed in PBS, and stained with 100 µM acrinidine orange for 10 min. Cells were washed in PBS and resuspended to a concentration of 3×10^7 cells/mL as determined by use of the Scepter handheld automated cell counter (EMD Millipore, Billerica, MA). To 7 µL of a 5% solids suspension of MHC II-conjugated microparticles was added 7 µL of SEB toxin and 1 µL of stained Jurkat cells. HeLa cells were first treated with trypsin and EDTA for 5 min at 37°C, then similarly washed, fixed, stained, and resuspended at a concentration of 3.8×10^7 cell/mL. 1 µL of HeLa cells were added to 7 µL of a 5% solids suspension of MHC II-conjugated microparticles and 7 µL of SEB toxin. Resultant bead pellets were imaged on a fluorescent microscope using 488 nm excitation and 525 nm emission. For BoNT/A, a His-tagged SNAP-25-GFP fusion protein construct was attached via immobilized nickel ions on a nitrilotriacetic acid-decorated microparticle. Following immobilization, EDC/NHS was used to crosslink the protein to the bead. For multiplexed activity and immunoassays, ricin was first incubated with both the silica capture particles and the PMMA-conjugated stem loop substrate for 20 min. Fluorescently-labeled APE1 and the detector antibody were then added to the suspension in PBS and allowed to incubate for 20 min. The suspension was then layered on top of the density medium, spun for 45 s at 8000 rpm, and imaged as above, using the 705 nm emission for the immunoassay and the 585 nm emission for the activity assay. Simultaneous SEB immuno- and activity assays used polystyrene-antibody conjugates for the upper layer. Micrographs showing representative images for Jurkat with and without SEB as well as the HeLa negative control are shown in Figure S4.

For assays conducted in food matrices, liquids such as juices and milk were used as received. Solid foods such as vegetables and meat were resuspended in buffer and homogenized with a mortar and pestle prior to spiking with the analyte of interest. Total hydrated food mass was kept constant at 25 wt%. ELISA protocol

ELISA protocol

Commercially available ELISA kits for shiga-like toxin, SEB, and ricin were used as a standard for method comparisons. ELISAs were performed according to manufacturer's instructions. Briefly, pre-coated plates were blocked with blocking buffer (5% (w/v) nonfat dry milk, 0.1% (v/v) Tween-20 in PBS, pH 7.4), sealed, and incubated for 1 h at 37°C. Plates were washed with PBST (0.1% (v/v) Tween-20 in PBS,

pH 7.4). Serial dilutions of the antigen were created in triplicate across the plate for the standard curve and six additional values were chosen between points on the standard curve for method comparison. Plates were sealed, incubated at 37°C for 1 h, and washed with PBST. The detector antibody was added at a concentration of 10 μg/mL, incubated for 1 h at 37°C, and the plates were washed with PBST. The conjugate antibody was thawed at room temperature for 5 min, diluted 1:5000, and added to the plates. Plates were sealed, incubated for 1 h at 37°C, and washed with PBST. Equal volumes of the A solution and B solution of the ABTS 2-part peroxidase substrate (KPL, Gaithersburg, MD) were mixed immediately prior to use. Plates were incubated at 37°C for 30 min. Plates were read on a Molecular Devices (Sunnyvale, CA) SpectraMax 110 plate reader at 410 nm. Representative method comparison plots are shown in Figure S5.

Results and Discussion

SpinDx Immunoassay

SpinDx immunoassay scheme is simpler compared to a typical microtiter plate-based immunoassay. The semi-automated protocol limits manual intervention to simply mixing the sample (clinical, environmental, or food) with beads and pipetting it into the disk and loading the disk in a reader. Briefly, an equal volume of the sample is mixed and incubated at room temperature with a suspension comprising capture antibody-functionalized beads (1-μm silica microparticles) and an unbound fluorescently-labeled detection antibody. The sample/detection suspension is loaded on top of a pre-loaded density medium in a channel embedded on the disk. The disk is loaded into the reader and spun using a rotary motor. During centrifugation, the microparticles carrying the antibody-antigen-labeled antibody complex sediment through the density medium and pellet at the bottom of the channel (periphery of the disk) while the other sample components and excess labeled antibodies remain in the loading chamber. The fluorescence of the microparticle pellet is measured to quantify concentration of the target analyte in the sample. The entire assay requires less than 30-min (compared to several hours for other *in vitro* assay approaches or days for the live-mouse bioassay). Furthermore, the scale of the device allows for small samples sizes (2-μL per sample), whereas other assays typically use much larger volumes (100-μL for ELISA or 500-μL for the mouse bioassay). An overview of the centrifugal sedimentation assay protocol is shown in Figure 1, depicted for multiplexed analysis of a drop of whole blood.

Detection of biotoxins in SpinDx

We focused on demonstrating the suitability of SpinDx platform to detect the key biotoxins of interest for biological terrorism include botulinum toxin A, botulinum toxin B, anthrax toxin protective agent (PA), ricin, SEB, and shiga toxins. We demonstrate here ultra-sensitive and rapid immunoassays for direct detection of a number of biotoxins, with demonstrated results in exogeneous human as well as both exogeneous and endogeneous animal samples (Figure 3). Assays feature wide dynamic range and resolution. For BoNT/A in serum, the median dynamic range (defined as the range over which 2-fold changes in concentration can be distinguished at 95% confidence) is from 6.7 fM – 10 nM (0.1 pg/mL – 10^6 pg/mL) with a 1.3-fold median concentration change discrimination. For BoNT/B in serum, dynamic range was achieved from 530 fM – 1 nM (8 pg/mL to 10^5 pg/mL) with a limit of detection of 40 fM (6 pg/mL). For ricin toxin, dynamic range was from 8.8 pM – 100 nM (68 pg/mL to 10^6 pg/mL) with a limit of detection of 1 pM (60 pg/mL). For SEB, dynamic range was from 380 pM – 1 μM (862 pg/mL to 10^4 pg/mL) with a limit of detection of 84 pM (201 pg/mL). For shiga-like toxin 1, dynamic range was from 5.7 pM – 800 nM (62 pg/mL to 10^6 pg/mL) with a limit of detection of 0.8 pM (56.4 pg/mL). While we demonstrate assays for selected toxins, the diagnostic approach is highly adaptable to additional analytes as the assay reagents (i.e., the capture beads and detection antibodies) are disconnected from the disk architecture, facilitating rapid development of new assays. The current disk architecture allows detection of up to 64 analytes simultaneously from one sample.

SpinDx Assays are ultra-sensitive

High sensitivity is a primary requirement for diagnosing exposure or intoxication from the priority toxins due to their extreme toxicities. We have achieved sensitivities that greatly surpass conventional diagnostic methods (for example, the ~500aM (.075 pg/mL) LOD for BoNT/A is ~100-fold more sensitive than the mouse bioassay (ref) and current ELISA protocols employed by the USDA and NBACC. The enhanced sensitivity of the SpinDx™ assay is attributed to several unique features of the sedimentation approach, including: a) 1-μm beads provide a capture surface ~320x larger than standard 96-well microtiter plates; b) isolating the capture beads from the sample and excess label during the sedimentation step inherently washes the beads with several hundred times the particle volume due to Stokes flow and significantly reduces the background signal without requiring separate wash steps; c) pelleting the beads at the bottom of the channel permits averaging of signal over hundreds of beads; and d) the use of quantum dots as the detection label provides a large (300-nm) Stokes shift thereby further reducing autofluorescence and background noise. Quantum dots are also resistant to photobleaching, allowing for longer signal acquisition times.

No off-device sample preparation is required for environmental or clinical samples

A key advantage of our technique is its ability to process complex samples, both environmental and clinical, with no additional sample preparation thus drastically reducing the duration and complexity of the assays. Complex samples can be mixed directly with the capture beads and detection reagents. Beads pellet out of the sample upon centrifugation, while other unbound particles (including cells, proteins, lipids, white powder components, food and other components) remain in the sample chamber. We have attempted immunoassays of analytes spiked in a large variety of samples including water, food, juice, milk, white powder, serum, saliva and whole blood and found minimal interference in assay performance. Figure 4 shows results from direct analysis of several important clinical and food sample matrices. BoNT/A was detected in clinical matrices such as whole blood, serum, and saliva with minimal matrix interference compared to the response from buffered systems alone. Food samples such as milk, canned meat, canned vegetables, juice, and salad dressing were also compatible with minimal deviation from a linear relationship. Foods that have low pH and are minimally cooked are most vulnerable to botulinum toxin contamination.[19-21] The two major outbreaks of BoNT/A intoxication in the United States were traced to canned green beans and carrot juice.[18, 22, 23] The

detection suspension is buffered by PBS, minimizing the effect of acidic sample matrices such as fruit and vegetable juices. Figure 4 shows photographs of the on-disk detection channels following assay completion with contaminants such as plasma and cells from blood, caseins from milk, and lipids from peanut butter isolated from the bead pellet by the density medium. However, while the food-based detection results in Figure 4 were obtained using spiked BoNT/A holotoxin, the toxin is often found in its natural complex form in environmental and food samples (and subsequently dissociated following intestinal absorption). Therefore, we also include dose-response quantification of hemagglutinin 70 (HA70) – a protein found in the BoNT/A complex - using HA70-specific monoclonal antibodies (see Figure S2 in Supplementary Materials). New assays are easily developed upon the SpinDx^{TM} platform by simple substitution of sandwich immunoassay affinity reagents.

SpinDx is highly-multiplexed and adaptable

A key limitation of existing lateral-flow assay, in addition to their lower sensitivity, is that they only detect one toxin at a time. Even the standard ELISAs (e.g., NBACC or USAD ELISA) only look for one toxin at a time. The gold-standard mouse bioassay is very sensitive but cannot differentiate between serotypes which is crucial to guide administration of the correct monoclonal antibody therapy. Our diagnostic approach is highly multiplexed and capable of measuring a number of targets (up to 64 parallel assays using the current disk architecture) within minutes in a single sample. On-disk multiplexing is achieved spatially via routing samples from a single inlet port to 36-64 individual assay channels for parallel processing of a single sample. Spatial multiplexing avoids issues with cross-reactivity encountered in intra-channel multiplexing schemes. SpinDx is also highly adaptable as the assay reagents (i.e., the capture beads and detection antibodies) are disconnected from the disk architecture, facilitating rapid development of new assays. This is crucial in cases where new serotypes of a toxin are discovered for example, the recent report on identification of a new botulinum serotype[24, 25]. We have demonstrated multiplexed detection of ricin, shiga, and SEB from the same sample on the same disc.

Detection of ricin in surrogate white powder samples

As illustrated by multiple incidents of discovery of ricin in letters sent to political leaders, there is a great need for rapid and sensitive detection of ricin and other toxins in white powder samples. White powders represent a challenging class of materials upon which to perform assays. Many parameters such as pH, viscosity, solubility, and background can vary greatly depending on the carrier powder. A significant advantage the SpinDx platform brings to this problem is the ability to isolate the signal from any interfering compounds via the density-based separation. For these experiments, white powder surrogates were compounded from common, commercially available substances. Milk powder, magnesium silicate, and sucrose were mixed to form the white powder. Dilutions of ricin toxin were created by adding stock solutions to the powders. To these powders were added PBS to dissolve or suspend the powders and to allow for pipetting onto the SpinDx platform. Microliter samples from the resultant samples were analyzed on the platform, demonstrating detection in white powder from 1 pM to 1000 pM.

Toxin activity assays

In addition to determining the presence of pathogenic bacteria and the presence of the toxin proteins, assessment of the activity of the toxin proteins is important for accurate diagnosis of afflicted patients. To this end, we developed assays for the activity of four toxins: BoNT/A, ricin, SLT, and SEB. Stem-loop substrates, inspired by sequences found in literature, were designed with the sarcin-ricin loop, a sequence highly conserved across 28S eukaryotic rRNA.[26] The toxins act by specifically depurinating a single adenine (A4324) in the sarcin-ricin loop preventing the binding of elongation factors and thus halting protein synthesis.[27] In order to detect the depurination event, we used fluorescently-labeled APE1 to bind specifically to abasic sites found in the substrate. Typically, APE1 participates in the DNA base excision repair pathway by nicking the phosphodiester backbone through acyl substitution mediated by a stabilizing Mg2+ ion after a DNA glycosylase removes a damaged base.[28] For this activity assay, tight binding of the enzyme was desired without the enzyme completing turnover; this was accomplished by depriving the enzyme of Mg2+ ions. A DNA substrate was chosen rather than an RNA substrate due to the ability of the toxins to act upon DNA[29, 30] as well as the greater stability of DNA compared to RNA. By covalently attaching the synthetic DNA stem-loop substrate to PMMA microparticles and then detecting the activity of the toxin upon these substrates by the use of APE1, we developed an activity assay amenable to the platform as shown in Figure 4A. Increasing the incubation time to 240 min increases sensitivity by approximately ten-fold (Figure 4A). SEB, a superantigen, acts by crosslinking the TCR to the MHC II causing acute stimulation of the immune system.[31] To assay for the activity of SEB, we used an immortalized T-cell line, Jurkat cells, which express the TCR on the cell surface.[32] MHC II was conjugated to 1 μm silica microparticles. Placed together in the presence of SEB, the silica microparticles act as a sink on the cells, driving them through the density medium as seen in Figure 4B. In the absence of the SEB, the cells stay above the density medium.

Other activity assays for ricin and shiga have been reported which include mass spectrometric-based assays[29, 33, 34], monitoring the depurination of adenine from ribosomes or synthetic constructs by HPLC[35], detecting the decrease in luciferase production from in vitro translation[36, 37], or through clever use of a cascade of enzymes to produce ATP-driven luminescence[38]. While several of these assays are both sensitive and specific, the assay architecture lends itself to an established laboratory setting. Mass spectrometers, HPLCs, and multiple complex liquid handling steps are not amenable to a fieldable assay. To date, this is the first SEB activity assay reported in the literature to the best of the authors' knowledge.

Multiplexing several targets to the same toxin protein is a method to improve confidence in the assignment of identity. To that end, we have performed in-channel simultaneous immuno- and activity assays for the toxin proteins. As demonstrated by fluorescent micrographs in Figure 5a, two color detection of

toxin can be achieved with good spatial and spectral separation between the immunoassay and activity assay – an additional advantage to using a centrifugal sedimentation-based platform (original micrographs can be found in the ESI, Figure S6). By determining the fraction of toxin which retains enzymatic activity in addition to the total amount of toxin protein present, a more accurate assessment of the danger to public health can be made.

SpinDx is a portable, easy-to-use instrument

Our overarching goal is to develop a rapid, reliable, cost-effective detection device (*SpinDx*) for use in primary care facilities, public health labs, and field-laboratories designated for testing of environmental samples. While SpinDx can process both clinical and non-clinical samples, we anticipate two versions of the device for regulatory reasons. We envision SpinDx as a simple-to-use device (Fig 1) in which manual intervention is limited to introducing the sample into a disk, loading the disk into a reader, and hitting the start button. The entire operation of sample metering, incubation with antibody-coated beads and labeled secondary antibodies, separation of beads from sample, and scanning of fluorescence is automated. The *SpinDx* software and barcode scanner will easily manage data and display results. The device has a small footprint weighing less than 5-lb, making it suitable for easy deployment in remote facilities. The device is adaptable to new assays as the reader is separate from the assay "kit" that includes an assay disk with pre-loaded reagents and sample collection tools.

Conclusions

The proposed SpinDx platform meets the stringent sensitivity and diagnostic time window requirements for effective treatment of individuals exposed to biotoxins. Our system is designed for direct analysis of samples (blood, urine, white powder, etc.) with no additional sample prep required and diagnostic results in less than 15 min. Unique signal enrichment and background suppression elements inherent to the assay approach enable sensitivities unmatched by conventional approaches. But perhaps key to the assay's successful adoption by its anticipated end-users and market is its generic architecture and adaptability to many clinical tests with minimal assay development effort. By this characteristic the proposed diagnostic system not only meets an urgent unmet need for biodefense, but also provides revolutionary instrumentation and capabilities for the public health community.

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Notes and references

[a] Biotechnology and Bioengineering, Sandia National Laboratories, Livermore, CA 94551, USA, E-mail: ckoh@sandia.gov, aksingh@sandia.gov.

[b] Currently at Cepheid, Sunnyvale, CA

[c] Currently at Sandstone Diagnostics, Livermore, CA

[d] Western Regional Research Center, Foodborne Contaminants Research Unit, U.S. Department of Agriculture – Agricultural Research Service, Albany, CA 94710

[e] University of Massachusetts Dartmouth, North Dartmouth, MA 02747

Electronic Supplementary Information (ESI) available: Detailed materials, synthetic procedures, and assay methods are available in the ESI. See DOI: 10.1039/b000000x/

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