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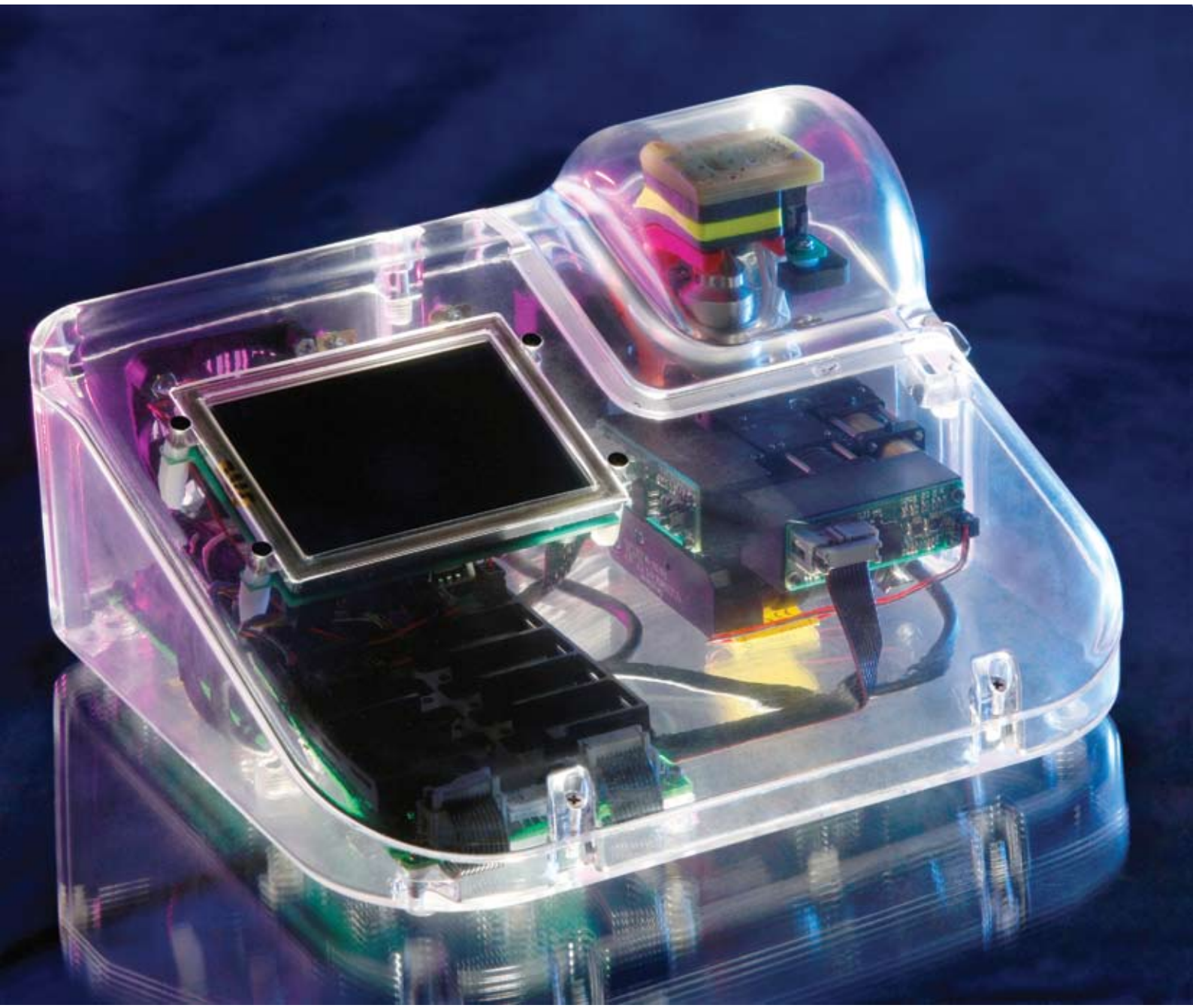

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Point-of-care Microfluidic Diagnostics

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An integrated microfluidic platform for sensitive and rapid detection of biological toxins†

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Towards designing a portable diagnostic device for detecting biological toxins in bodily fluids, we have developed microfluidic chip-based immunoassays that are rapid (< 20 minutes), require minimal sample volume (<10 μL) and have appreciable sensitivity and dynamic range (μM–pM). The microfluidic chip is being integrated with miniaturized electronics, optical elements, fluid-handling components, and data acquisition software to develop a portable, self-contained device. The device is intended for rapid, point-of-care (and, in future, point-of-incident) testing in case of an accidental or intentional exposure/intoxication to biotoxins. Detection of toxins and potential host-response markers is performed using microfluidic electrophoretic immunoassays integrated with sample preconcentration and mixing of analytes with fluorescently labeled antibodies. Preconcentration is enabled by photopolymerizing a thin, nanoporous membrane with a MW cut-off of ~10 kDa in the sample loading region of the chip. Polymeric gels with larger pores are located adjacent to the size exclusion membrane to perform electrophoretic separation of antibody–analyte complex and excess antibody. Measurement of the ratio of bound and unbound immune-complex using sensitive laser-induced fluorescence detection provides quantitation of analyte in the sample. We have demonstrated electrophoretic immunoassays for the biotoxins ricin, Shiga toxin I, and Staphylococcal enterotoxin B (SEB). With off-chip mixing and no sample preconcentration, the limits of detection (LOD) were 300 pM for SEB, 500 pM for Shiga toxin I, and 20 nM for ricin. With a 10 min on-chip preconcentration, the LOD for SEB is <10 pM. The portable device being developed is readily applicable to detection of proteinaceous biomarkers of many other diseases and is intended to represent the next-generation diagnostic devices capable of rapid and quantitative measurements of multiple analytes simultaneously.

Introduction

As people, goods, and information move across the globe at an ever-increasing pace, the chances of a successful intentional or accidental biotoxin exposure are increasing. For most biological toxins such as ricin, Staphylococcal enterotoxin B (SEB), Shiga toxin, and botulinum, prognosis is poor once the distinguishing clinical signs manifest,[1,2] making early diagnosis critical for effective treatment of toxin exposure. For SEB, toxin is transferred into circulation within 30 to 90 minutes after inhalation exposure[3] and toxicity is observed as early as 4 hours post-exposure from superantigenic effects of the toxin including high cytokine production. 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. Microscopic damage to lung tissue may occur by 8–12 hours following inhalation exposure to ricin, and irreversible biochemical changes may occur within 60 to 90 minutes,[4] even if outward signs of intoxication do not appear until 12–24 hours post-exposure. Shiga toxin intoxication is less well understood, but the toxins have a mechanism of action similar to ricin,[5] and the need for a rapid diagnostic is apparent.

Medical devices to rapidly diagnose (or triage) victims of a release of biological toxins are clearly critical for effective countermeasures. A rapid and portable diagnostic method will be useful in many scenarios including facilities with large, transient populations (*e.g.* transportation hubs and government buildings), and populated venues such as sporting events where the quick screening of a large number of people is vital. These next-generation devices need to be rapid, cost-effective, specific, and ideally capable of detecting pre-symptomatic markers to enable effective countermeasures including therapeutic intervention at the earliest stages of intoxication. Such systems must also be easy-to-use, automated, rugged, self-contained and preferably, have a small footprint to allow use in point-of-care and point-of-incident settings. The device must be cost-effective so that a large number of people can be screened, though robust enough to provide a reliable diagnosis.

The need for early detection of toxin exposure is clear, but many of the current diagnostic methods are simply inadequate for this task. Conventional bench-top methods (*e.g.*, enzymatic activity assay, animal assay, ELISA) are limited by expense, labor demands, long assay times, and the need for highly trained personnel. The detection of antibodies to toxin in human blood samples provides a sensitive and specific diagnostic method, but the approach is limited to detection of seroconverted diseases

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(often 3–20 days post-exposure). The presence of antibody is also not conclusive for some toxins including SEB; most human serum contains antibody that binds SEB and other pyrogenic toxins, meaning titer tests have little diagnostic value.[6] DNA-based diagnostics are less meaningful in the face of a bioterrorism incident where victims may be exposed to purified protein toxin or infected by genetically modified microorganisms. An approach that directly identifies the toxin can be performed earlier than a method that relies upon seroconversion and is not confused by prior environmental exposure.

Advances in microtechnologies[7–9] are revolutionizing many areas of biological analysis, including genomics,[10,11] proteomics[12,13] and clinical diagnostics.[14,15] The portability, speed of analysis, low sample and reagent consumption, and potential for automation and integration make microfluidic technologies well-suited to point-of-care diagnostic instrumentation. Electrophoretic immunoassays represent one promising technique for highly specific detection of protein biotoxins in a microfluidic format. Capillary electrophoresis has previously been demonstrated as an efficient means to separate immune complex from free antibody or antigen.[16–19] In such systems, a sample which may contain an analyte of interest is mixed with a specific detection antibody. Electrophoresis allows the high-resolution separation of free antibody from the antibody–antigen complex, based on differences in the electrophoretic mobilities (or charge-to-friction force ratios) of the two species. A complete description of electrophoretic immunoassays may be found in recent reviews and references therein.[20–22] Other affinity reagents, such as aptamers, have also been used instead of antibodies in similar types of mobility-shift electrophoretic assays for targets including ricin.[23]

Attaining adequate species discrimination with electrophoresis-based immunoassays can be challenging. Large analytes such as antibodies and immune complexes vary little in electrophoretic mobility.[20] Techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) allow excellent discrimination of species by size, but SDS disrupts fragile immune complexes, making quantitation of complexes impossible. Native (non-denaturing) PAGE techniques, both with and without detergent, have been shown to retain the biological activity necessary for intact immune complexes, yet provide sufficient size-based sieving to allow analyte discrimination.[24]

Specific advantages of microdevice-based electrophoretic immunoassays include the potential for shortened incubation times (as compared to solid-phase immunodiagnostic systems such as ELISA), simplified assay protocols (as compared to the multiple wash and detection steps required for conventional immunodiagnostics), and device form-factors amenable to system integration and automation. Several groups have previously demonstrated elegant architectures for conducting microscale immunoassays based on electrophoretic separation[14,15,25–28] and other techniques (*e.g.* solid-phase assays).[29–34] Such demonstrations hold promise for development of automated, high-throughput systems for medical diagnostics.[35]

Our group has significant expertise in translating microfluidic assays from an optical bench into a truly portable, miniaturized format that can be deployed almost anywhere. Recent prototypes designed and tested include the Integrated Microfluidic Platform for Oral Diagnostics, or IMPOD, designed for point-of-care detection and quantitation of protein biomarkers of periodontal disease in saliva using an electrophoretic immunoassay[36,37] and the MicroChemLab, a handheld portable device for separation and analysis of biological agents.[38–41] Leveraging these earlier prototypes, we report on development of a portable diagnostic device for detection of biotoxins, beginning with immunoassays for SEB, ricin, and Shiga. The device utilizes glass chips for microchannel electrophoresis in a portable, self-contained device with integrated electronics, miniaturized optics (diode lasers, mirrors, lenses, filters, and PMT), fluid handling components, and data acquisition software. It performs rapid microfluidic chip-based immunoassays (3–20 min) with nanomolar to picomolar sensitivity. The microfluidic chip incorporates multiple photopatterned polyacrylamide gel elements, facilitating sample filtration, target enrichment, on-chip mixing of sample with detection antibody, and electrophoretic separation. The electrophoretic immunoassay platform is highly flexible, and can readily be adapted to a wide variety of protein targets. We report here on the development of immunoassays for new protein biotoxin targets and the development of a novel integrated device.

Materials and methods

Microfluidic chip fabrication

Microfluidic chips for electrophoresis were fabricated in glass using standard photolithography and wet etch techniques.[42] Microchannel patterns were designed using AutoCAD software, and a high-resolution chrome mask with 20 μm wide features was prepared by Photosciences (Torrance, CA). Photolithography, single-level etch, bonding, and dicing were performed by Caliper Life Sciences (Mountain View, CA). A 30 μm isotropic etch resulted in channels 80 μm wide at the top, and 20 μm wide at the bottom.

Two microchannel layouts were used for electrophoresis, illustrated schematically in Fig. 1. For experiments with off-chip mixing of reagents, a simple "double-T" layout (100 μm offset) was used for electrophoresis. The entire microchannel was filled with a photopolymerized crosslinked polyacrylamide gel (6% T, 5% C, where % T refers to the total monomer mass concentration, and % C refers to the mass of crosslinker as a percentage of the total monomer), following a procedure described previously.[43] Briefly, the surface was first treated with 3-(trimethoxysilyl)propyl methacrylate (Aldrich, St. Louis, MO) to provide a point of attachment for the polyacrylamide gel. Next, the microchannel was filled with a solution of acrylamide monomer, *N*,*N*-bisacrylamide crosslinker, and VA-086 photoinitiator in 1× Tris-Glycine buffer (BioRad, Hercules, CA). The solution was polymerized upon illumination for 10–15 minutes from a 100 watt UV lamp or a Spectrolinker XL 1500 UV oven with 365 nm tubes. Gel-filled chips were stored in 1× Tris-Glycine buffer at 4 °C when not in use. Operation of the double-T chip was similar to previously described procedures.[43]

For experiments with integrated preconcentration and reagent mixing on-chip, a more complex layout with separate reservoirs for sample and antibody, and multiple buffer and waste reservoirs was used, as illustrated in Fig. 1B and as described

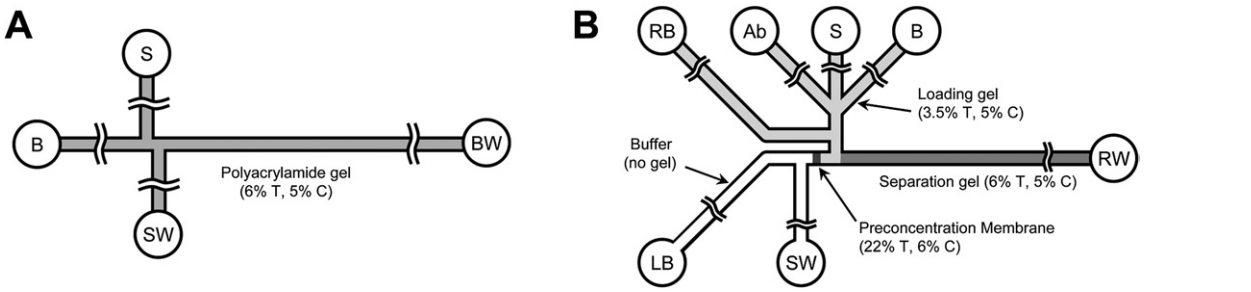

Fig. 1 Schematic diagram of microchannel layouts. (A) Simple "double-T" chip design for electrophoresis with off-chip mixing of reagents. S = sample, SW = sample waste, B = buffer, BW = buffer waste. Approximate length from B to BW is 4 cm; from injection zone to BW is 2.9 cm. The detector was typically positioned 5 mm from the injection zone. (B) Microchannel layout for experiments with integrated sample preconcentration and reagent mixing; see ref. 37 for greater detail. S = sample, SW = sample waste, Ab = antibody, B = buffer (not used here, but could hold a second sample or antibody), LB = loading buffer, RB = running buffer, RW = running waste. Photopatterning was used to define multiple gel regions with different acrylamide concentrations as shown in the figure. The LB well is filled with a larger volume of buffer than the SW well, providing a continuous hydrodynamic flow of buffer past the "downstream" side of the membrane.

previously.[37,42] Gel fabrication was similar to that for the simple double-T chips described previously, except that three sequential polymerization steps with different acrylamide concentrations were used to define different zones. First, a nanoporous polyacrylamide size exclusion membrane (22% T, 6% C) was defined by photopolymerization with shaped UV laser light (355 nm frequency-tripled Nd : YAG, projected through a slit). Subsequently, a separation gel (6% T, 5% C) was defined in the main separation channel by flood illumination through a photomask. Finally, a low-concentration loading gel (3.5% T, 5% C) was defined in the region between the sample and antibody reservoirs and the preconcentration membrane. The region downstream of the preconcentration membrane was left open (no gel).

Ricin immunoassay

Ricin (Ricinus Communis Agglutinin II, RCA60) and goat polyclonal antibody to ricin (Anti-Ricinus Communis Agglutinin I and II) were purchased from Vector Laboratories (Burlingame, CA, USA). The antibody was fluorescently labeled by reaction with Alexa Fluor 546-NHS ester (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The labeled antibody was purified from unreacted dye using a two-step purification: concentration with a Microcon spin ultrafiltration device (Millipore, Billerica, MA, USA), followed by size-exclusion chromatography in a Centri-Sep spin column (Princeton Separations, Adelphia, NJ, USA). The degree of labelling was determined spectrophotometrically to be 4–5 labels per antibody.

Immunoassays were conducted in 1× Tris Glycine buffer (BioRad, Hercules, CA, USA), or in fetal bovine serum (Invitrogen) diluted 1 : 10 in 1× Tris Glycine buffer. 15 nM of labeled anti-ricin was mixed (off-chip) with ricin at concentrations ranging from 5 nM to 970 nM. BSA-Alexa Fluor 555 conjugate (Invitrogen) was used as an internal standard for each reaction, at a final concentration of 2.3 nM. Reactions were carried out in polypropylene microcentrifuge tubes, which had been passivated by incubating overnight with a 1 mM solution of BSA. The immunoassay reactions were incubated at room temperature for >30 minutes, and then transferred to the microfluidic chip for electrophoretic analysis.

Native gel electrophoretic analysis was carried out in simple "double-T" chips described above. The chips were mounted in a custom Delrin chip holder with 2 mm diameter fluidic reservoirs and an aluminium compression frame. A custom-built miniaturized high-voltage power supply with eight independent channels[40] was used to drive electrophoresis, *via* a LabView interface providing direct control over voltages, as well as feedback control of current. External platinum electrodes were used to address the fluidic reservoirs. Proteins were transported electrophoretically from the sample well to the sample waste well by applying a field of 200 V/cm between the sample well and the sample waste well for 3 minutes, sufficient to fill the injection zone between the offset-T arms of the chip. For injection and separation, a field of 225 V/cm was applied between the buffer well and waste well, with a "pullback" voltage applied to the sample and sample waste wells for the first 20 seconds following injection. The detector was positioned 5 mm from the sample injection zone. At least six repeat injections of each sample were performed.

On-chip laser-induced fluorescence detection was accomplished using a home-built confocal LIF setup with an optical chopper and lock-in amplifier, similar to that described previously.[43] For the Alexa Fluor 546/555-conjugated proteins, excitation was accomplished with a 532 nm laser (frequency-doubled Nd : YAG, Melles-Griot, Carlsbad, CA, USA), with a 560DRLP dichroic reflector (Omega Optical, Brattleboro, VT), and a 560–610 nm bandpass emission filter (3rd Millenium, Omega). Fluorescence emission was detected with a PMT module (Hamamatsu 5784-20) with a home-built low-voltage power supply. The chip holder was affixed to an *xyz*-translational stage, allowing precise positioning of the laser focus inside the microchannels.

Shiga toxin I immunoassay

Immunoassays for Shiga Toxin I were performed using off-chip mixing in a manner similar to that described for ricin. Shiga toxin 1 (Stx1) and murine monoclonal antibody (3C10) to Shiga toxin I (anti-Stx1) were purchased from Toxin Technology, INC (Sarasota, FL, USA). The antibody was fluorescently labelled by reaction with Alexa Fluor 647-NHS ester, and purified as

described previously. Detection of fluorescent antibody and antibody–toxin complex was accomplished using a confocal LIF setup employing a 633-nm HeNe laser and long-pass emission filter, but otherwise substantially similar to the 532 nm LIF setup described previously.

Staphylococcal enterotoxin B (SEB) immunoassay

Immunoassays for SEB were performed using both off-chip mixing, and on-chip mixing with preconcentration. SEB and murine monoclonal antibody (anti-SEB, MB87) were purchased from Toxin Technology, Inc. The antibody was fluorescently labelled by reaction with Alexa Fluor 647-NHS ester.

Immunoassays for SEB were performed both with off-chip mixing (as described above for ricin), and also with on-chip sample preconcentration and reagent mixing. Detailed discussion and a schematic of the on-chip preconcentration and mixing procedure can be found in ref. 37. A simplified run sequence involves first loading and preconcentrating antibody at the membrane interface with a field of 50 V/cm for 1 minute. This is followed by concentrating proteins from the sample well against the preconcentration membrane at low voltage (20 V/cm) resulting in rapid mixing of sample proteins with antibody within a narrow zone adjacent to the membrane interface. Sample proteins were concentrated for 10 minutes. Finally, the concentrated zone of sample protein and antibody is mobilized into the main separation channel for electrophoretic separation at 250 V/cm. To minimize concentration polarization effects, the field is applied for a short (15 s) duration across the membrane with a pinching field from the buffer reservoir, and then for the remainder of the separation the membrane is bypassed.

Safety note

Ricin, Shiga toxin, SEB, and other biotoxins are hazardous materials that may be lethal when ingested, inhaled, or injected in nanogram or lower doses. Appropriate precautions must be taken for storage, handling, and disposal of toxins and contaminated materials. Work should be performed in compliance with all relevant federal and local regulations governing these substances. Experiments were performed in a BioSaftey Level 2 (BSL2) laboratory.

Results and discussion

Immunoassays with off-chip mixing

We report here for the first time application of our previously developed microfluidic electrophoretic immunoassay with photopatterned gel elements[37,43] to the detection of SEB, ricin, and Shiga toxin. Immunoassays for Ricinus Communis Agglutinin II, Shiga Toxin I, and Staphylococcal enterotoxin B were first performed with off-chip mixing of toxin samples and antibodies, followed by microchip native PAGE analysis with fluorescence detection. A typical set of electropherograms for the ricin immunoassay is shown in Fig. 2. With no toxin present, the antibody migrates as a single peak, centered at 30 seconds, with a width of about 16 seconds (peak width is measured across the peak at one-half of the maximum height). With addition of ricin to the sample, a second broad peak appears, centered around 60 seconds, which represents the ricin-Ab immunocomplex. The complex peak increases in size with increasing amounts of ricin, while the antibody peak decreases in size. Immunoassays for SEB and Stx1 were similar, although the width of the complex peak and the degree of separation between the antibody and complex peak differs considerably for each toxin.

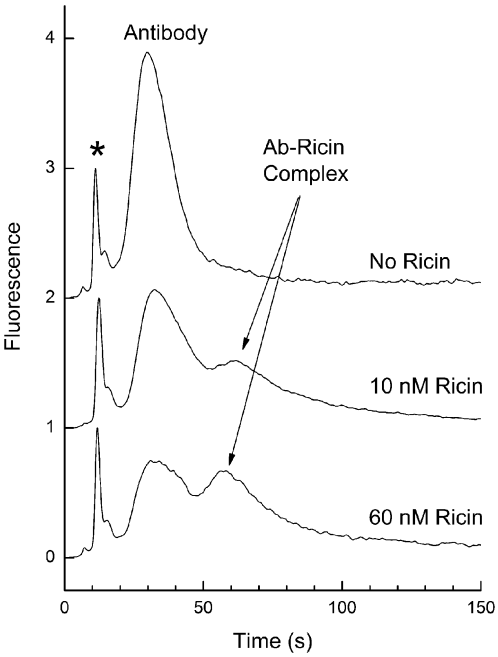

Fig. 2 Microchip electrophoretic immunoassay for detection of ricin. LIF detection was accomplished 5 mm downstream from the injection zone, with excitation at 532 nm, and detection centered at 590 nm. Each assay included 2.3 nM BSA labelled with Alexa Fluor 555 as an internal standard (*), and 15 nM of polyclonal anti-ricin IgG labelled with Alexa Fluor 546, in 1× Tris-Glycine buffer, spiked with varying amounts of ricin (whole toxin, RCA60). The separation was performed in a 6% T, 5% C polyacrylamide gel, at an electric field strength of ~250 V/cm. Fluorescent signals were normalized to the size of the BSA standard peak.

The native PAGE technique is particularly useful for situations where there is a significant difference in the sizes of the antibody and the complex. For moderate to large toxins (ricin is 66 kDa; Stx1 is 70 kDa), the sieving properties of the gel play an important role in the separation. For smaller antigens (*e.g.* SEB, 28 kDa), the antibody and the complex are closer in size, and the separation is based more on differences in the charge between the antibody and the complex. Even in such situations, the gel in the native PAGE technique offers the advantage of an anti-convective medium, preventing gravity-driven flow that frequently plagues open-channel electrophoresis in microfluidic chips. Sieving quality for smaller antigens can also be improved by increasing the total acrylamide concentration up to 8% T.

The areas of the antibody peak and complex peak can be measured (relative to the internal standard, fluorescently labelled BSA) to give a dose–response curve for the immunoassay, allowing quantitation of toxin in unknown samples. Dose response curves for SEB, Stx1, and ricin are shown in Fig. 3. The SEB and Stx1 assays give the expected sigmoidal shape, which can be fit to a logistical curve model, which is commonly used to model dose–response behavior with saturation at high

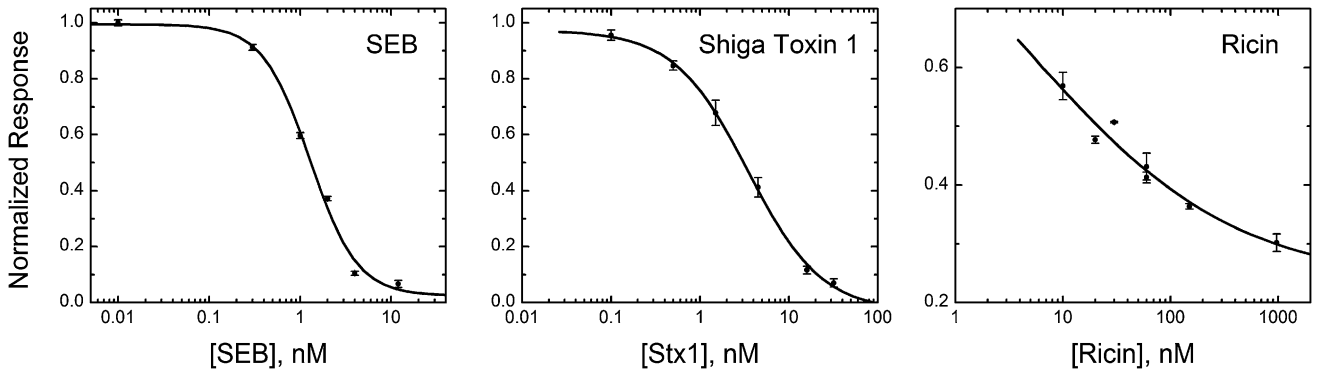

Fig. 3 Dose–response curves obtained for Staphylococcal enterotoxin B, Shiga Toxin 1, and Ricinus Communis Agglutinin II using electrophoretic immunoassays with off-chip mixing. Normalized response refers to the peak area or height of the antibody peak at a given toxin concentration, relative to the peak area or height with no toxin present. The normalized response for SEB and Shiga are based on antibody peak area, whereas the response for ricin is based on the antibody peak height. Error bars refer to the standard deviation of at least three runs. The solid curves are four-parameter logistical curve fits.

concentration. For the ricin immunoassays, the antibody peak and complex peak overlapped significantly, with long tails and were difficult to model accurately with simple peak shapes. In this case, the ratio of antibody peak height to the internal standard (BSA) peak height provided the best correlation to the ricin concentration. Each of the assays reveals a different dynamic range for detection, based on different affinities of the antibodies for their respective toxins. However, the preconcentration method described next allows us to achieve clinically relevant limit-of-detection with less dependence on the binding constant of antibodies.

Immunoassays with preconcentration

Low nanomolar detection limits are not always adequate for screening of biotoxins that may be lethal at picomolar or even lower concentrations. Taking advantage of our ability to photopattern polymeric elements,[44,45] we previously developed a novel on-chip preconcentrator[42] that uses a size-exclusion membrane fabricated in a channel to concentrate proteins bigger than the MW cut-off (~ 10 kDa) of the membrane. Two polymeric elements—a thin (~50 μm) size-exclusion membrane for preconcentration and a longer (~2 cm) porous monolith for protein separation—were fabricated by *in situ* photopolymerization. Contiguous placement of the different polymeric elements in the channels of a microchip enabled simple and zero-dead volume integration of the preconcentration with PAGE. Proteins are loaded through a low-concentration polyacrylamide gel, which does not significantly retard proteins in this size range, but which provides an important anti-convective effect. In addition, the low-concentration polyacrylamide loading gel excludes particles and very large macromolecules present in bodily fluids from entering the microchannels. In an immunoassay with preconcentration, protein biotoxins (as well as other proteins in the sample) in the sample are trapped and concentrated in a narrow zone on the upstream side of the size-exclusion membrane. In a second step, detection antibodies are trapped and concentrated in the same narrow zone on the upstream side of the membrane. The extent of protein and antibody preconcentration is easily tuned by varying the duration of preconcentration, or by controlling the sample volume loaded. During the preconcentration step, a continuous gravity-driven flow of fresh buffer past the downstream side of the membrane counteracts ion depletion or concentration polarization effects. Upon preconcentration, both the antibody and toxin are co-localized in a small volume (axial span <100 μm, volume ~10–100 fL) at high concentration, and the immunocomplex forms within seconds. Upon switching the direction of the electric field, the concentrated proteins are directed into the crosslinked polyacrylamide separation gel for electrophoretic analysis.

The electropherograms obtained with preconcentration appear similar to those obtained with off-chip mixing (*e.g.* Fig. 2), with an antibody peak and a complex peak along with an internal standard peak. The immunoassay with preconcentration offers simplified operation (by eliminating off-chip mixing steps), as well as significantly lower limits of detection. Fig. 4 illustrates the dose–response curve obtained for SEB with and without preconcentration. Preconcentration evidently offers greater than

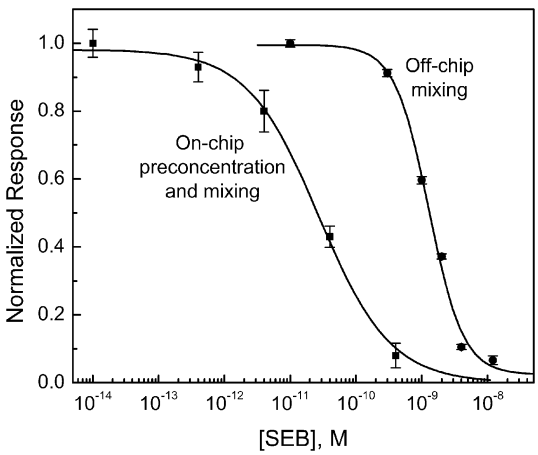

Fig. 4 Dose–response curves obtained for Staphylococcal enterotoxin B immunoassays with and without on-chip preconcentration and incubation. The solid curves are four-parameter logistical curve fits. The 10 minute preconcentration protocol shifts the dose–response curve ~2 orders of magnitude lower in concentration, with a larger dynamic range indicated by a more gradual downslope.

100-fold improvement in the limit of detection for this toxin, into the low picomolar range.

With detection limits <10 pM, the immunoassay with preconcentration compares favourably to current "gold-standard" approaches such as ELISA for direct detection of toxins. Sample volume requirements for the microfluidic immunoassay are significantly reduced compared to a conventional ELISA. The sample volume that is processed in a single preconcentration assay is typically on the order of the swept volume of the channel between the sample well and the preconcentration membrane (~20 nL) and thus several replicates of the assay can theoretically be performed with <1 μL of sample. The primary volume requirement is simply that enough liquid be present in each reservoir to fully immerse the electrode, and for the liquid in the reservoir to make electrical contact with the microchannel; typically this requires <10 μL of sample. The microchip immunoassay with on-chip preconcentration and mixing is at least an order of magnitude faster than ELISA, requiring <20 minutes per sample, with no user intervention required, apart from addition of reagents to fluidic reservoirs on the chip. This speed of detection is of critical importance for toxins such as SEB, which exhibit toxicity in less time than it takes to complete a typical ELISA. Since the preconcentration assay essentially consists of a series of simple electrophoretic steps, with no moving parts (valves, *etc.*), it represents a good candidate for implementation onto a multichannel array device, to achieve throughput that is competitive with assays performed in a multiwell microtiter plate.

Immunoassays in the current study were performed with two spectrally distinct fluorescent labels (Alexa Fluor 546 and Alexa Fluor 647), using two separate LIF setups. Thus far, the choice of fluorescent label has had little impact on performance of the immunoassay. Multiplexed immunoassays using multiple detection antibodies are possible using spectrally resolved fluorescence detection, which can readily be achieved with multiple inexpensive solid-state excitation sources, and multiple PMTs or CCD array-based detectors.

Selection of antibodies for immunoassays

Similar to ELISA or any other antibody-based assay, the selection and validation of the antibody is of the utmost importance in an electrophoretic immunoassay. Multiple antibodies are available for many toxins from several vendors; few are sold with any reliable data regarding the avidity of the antibody for the toxin, or the specific part of the toxin targeted by the antibody. Advances have been made in characterization of monoclonal antibodies, particularly those designed for human therapeutics,[46] but vendors of antibodies for *in vitro* diagnostics provide greatly different levels of quality control, sometimes with substantial lot-to-lot variability. Hence, we devoted a significant amount of effort and resources in testing and qualifying antibodies from many different vendors. This issue of quality control is not trivial—once an immunoassay is validated with a particular antibody, a reliable, large-scale source of that antibody must be identified before a portable diagnostic device can be widely distributed. Our preference is for monoclonal antibodies, as these provide better lot-to-lot consistency, and uniform (if not necessarily strong) binding of the target in 1 : 1 or 1 : 2 complexes. Polyclonal antibodies can be used as well, as demonstrated here for ricin, for which an appropriate monoclonal antibody was not identified among several commercially available candidates tested. Polyclonal antibodies may bind to the target in large, inhomogeneous complexes, which in an electrophoretic immunoassay may lead to a wide or tailing complex peak.

Development of a portable device for toxin detection

In parallel to the devlopment of immunoassays for multiple biological toxins, we have been developing an integrated device that will be used for multi-analyte toxin diagnostics. Fig. 5 shows the photograph of an early prototype being developed. The prototype incorporates electronics, optics, and chip architecture to enable multiplexing for higher throughput, which is critical for diagnostics following a suspected mass release of a toxin, with associated public demand for medical services placing a strain on medical infrastructure. The device is roughly the size of a desktop phone with dimensions of 23 cm wide × 20 cm deep × 13 cm tall, and weighs 5 lb. A touch-screen LCD provides for an improved, user-friendly interface, and an in-house designed miniaturized lock-in amplifier (approximately 3 cm × 4 cm × 12 cm) provides an estimated order-of-magnitude improvement in sensitivity for the LIF detection. Two excitation lasers and two detection channels allow simultaneous detection of antibodies labeled with spectrally distinct fluorophores. A disposable assay cartridge has been developed, with the chip, reagents, and electrodes packaged together, for one-step, snap-in-place replacement of the chip and reagents. The cartridge is amenable to low-cost mass production by injection molding. A miniaturized valve prevents pre-mixing of reagents with the on-chip gel during storage. An in-house-designed miniaturized syringe pump and microfluidic solenoid valve can be included for pressure-driven flow for on-board sample pretreatment. We plan to incorporate chromatographic removal of high-abundance serum proteins from blood samples into the portable device, although this technology is still under development. The biotoxin immunoassays presented in the preceeding section were performed using several components designed for the portable device (power supplies and high voltage

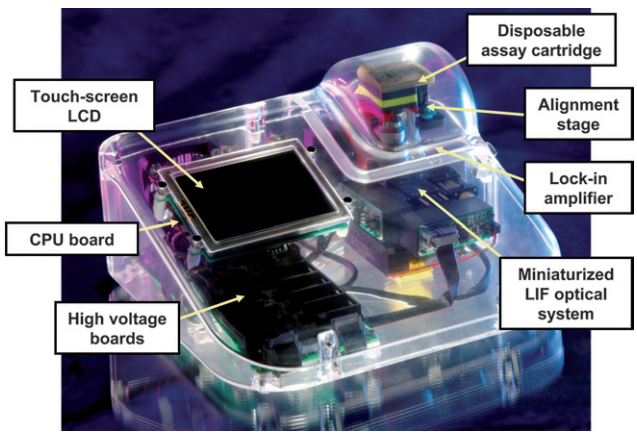

Fig. 5 Prototype of the portable device for toxin diagnostics in a clear acrylic case showing internal components. The device is approximately 23 cm wide × 20 cm deep × 13 cm tall, and is powered by a conventional 12V–1A DC power adapter (not pictured).

boards, electronics, optics, and software), but not packaged in the compact format pictured in Fig. 5. The miniaturized high voltage boards and the LIF optical system have been described in detail previously.[39,40]

We note that several other research groups have presented innovative strategies for rapid, direct detection of biotoxins in bodily fluids, with sensitivity reported in the femtomolar to nanomolar range. These strategies include micropatterned antibody arrays,[47] DELFIA,[48] immuno-PCR,[49] and a bead-based displacement immunoassay.[34] To our knowledge, no detection platform (ELISA or other strategy) is currently in widespread use for point-of-care detection for biotoxin exposure.

The portable device for point-of-care testing for toxin exposure needs to incorporate the following characteristics

(1) Accepts a readily available sample (blood serum, *etc.*), with minimal sample handling or preprocessing outside of the device.

(2) Small volume requirement, including the sample and all buffers, reagents, and waste streams.

(3) Capability for detection of multiple analytes.

(4) Simple user interface and autonomous control for sample-to-answer capability with minimally trained personnel.

(5) Rapid results allowing a speedy diagnosis and proactive treatment.

(6) Low cost per analysis, allowing testing of a large population of potential victims.

Manufacturability is a key concern for developing a low-cost device. In developing our immunoassays, the *in situ* gels are fabricated in small batches, 2–4 chips at a time. Laser patterning of the membrane is robust and reliable, and is successful nearly every time. Fabrication of the rest of the gel is less reliable when these devices are made in small batches. The majority of failures result from bubble formation in the gel during photopolymerization. The failure rate depends on the skill of the individual performing the fabrication, and ranges from 10–25%. In a larger scale manufacturing process, rigorous control over the gel polymerization (oxygen-free processing, precise control of temperature and radiation exposure, *etc.*) is expected to improve the success rate.

Long shelf-life of chips and reagents is an important criterion in development of our portable diagnostic device. In our experience, the gels and preconcentration membranes have remained effective for as long as we have tried storing a chip (on the order of 4–6 months), provided that the chip is kept wet, at 4 °C. Ideally, however, all components of the device should have a shelf life greater than 1 year. This includes not only the polyacrylamide separation gel and preconcentration membrane within the chip, but also the detection antibodies, and any reagents required for sample pretreatment. Refrigerated storage (4 °C) will be required to maintain the long-term stability of the antibodies. Several companies currently sell pre-cast polyacrylamide gels with shelf lives greater than 1 year at temperatures from 4–25 °C. Specific details of the gel and buffer compositions needed for long shelf life are usually proprietary, but likely include a buffer pH close to neutral to avoid slow alkaline hydrolysis of the polyacrylamide. Further testing and development is still required to determine the useful life of our device.

We are also evaluating the ability of our chips to analyze multiple bodily fluids. While blood serum will remain the primary sample, we also plan to evaluate nasal swabs as they may provide sufficient traces of toxin for detection following an inhalation exposure. A variety of methods are being evaluated for pretreatment of both blood and nasal swab extract, and we are currently working toward integrating these techniques into our prototype device to minimize the amount of time-consuming off-chip sample handling on a potentially infectious blood sample. Effective depletion of high-abundance serum proteins (albumin, immunoglobulins, *etc.*) is particularly important for assays based upon preconcentration of a low-abundance target, because all proteins larger than the molecular weight cutoff of the membrane (~10 kDa) are concentrated simultaneously (although not generally at the same rate). Thus, even a serum sample that has been depleted of a large amount of albumin may still prove problematic if the trace of remaining albumin is concentrated 100-fold. Commercially available ion exchange and immunodepletion kits serve as a useful starting point for assay development, but we anticipate that several novel approaches currently being developed in our laboratory will prove more compatible with the chip-based electrophoretic immunoassay, and will be easier to integrate with the photopatterned polyacrylamide gel architecture.

With a footprint less than 500 cm^2, the portable diagnostic device can sit on a countertop in a clinician's office. The device is constructed with interlocks and engineered controls to minimize the possibility for exposure to electrical, laser, chemical, and biological hazards. These features allow the device to be deployed in a relatively uncontrolled atmosphere such as a clinic or a remote location. The small footprint would also allow the device to be deployed in a highly controlled environment such as a biosafety 3 or 4 laboratory where space is at a premium.

Concluding remarks

Biological toxins and pathogens pose a serious threat to our society—mostly in the form of accidental exposure or bioterrorism incidents. To rapidly diagnose and treat people in suspected incidents, there is an urgent need for miniaturized devices that can detect the presence of toxins rapidly and sensitively with low false positives. We are currently testing a new portable microscale platform to enable rapid electrophoretic immunoassays for biotoxins, including on-board pretreatment of blood serum samples. The immunoassays presented here for SEB, Shiga Toxin, and ricin form the basis for a sensitive, portable diagnostic that can be rapidly deployed in case of mass exposure of a large population to a biotoxin weapon.

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