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Rapid detection of trace bacteria in biofluids using porous monoliths in microchannels

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ABSTRACT

We present advancements in microfluidic technology for rapid detection of as few as 10 rickettsial organisms in complex biological samples. An immuno-reactive filter, macroporous polyacrylamide monolith (PAM), fabricated within a microfluidic channel enhances solid-phase immuno-capture, staining and detection of targeted bacteria. Bacterial cells in samples flowing through the channel are forced to interact with the PAM filter surface due to size exclusion, overcoming common transport and kinetic limitations for rapid (min), high-efficiency (~100%) capture. In the process, targeted cells in sample volumes of 10 μl to >100 μl are concentrated within a sub-50 nl region at the PAM filter edge in the microchannel, thus concentrating them over 1000-fold. This significantly increases sensitivity, as the hydrophilic PAM also yields low non-specific immuno-fluorescence backgrounds with samples including serum, blood and non-targeted bacteria. The concentrated target cells are detected using fluorescently-labeled antibodies. With a single 2.0 × 2.0 × 0.3 mm PAM filter, as few as 10 rickettsial organisms per 100 μl of lysed blood sample can be analyzed within 60 min, as compared to hours or even days needed for conventional detection methods. This method is highly relevant to rapid, multiplexed, low-cost point of care diagnostics at early stages of infection where diagnostics providing more immediate and actionable test results are needed to improve patient outcomes and mitigate potential natural and non-natural outbreaks or epidemics of rickettsial diseases.

1. Introduction

The ability to accurately diagnose potentially lethal infectious diseases at or even before their early symptomatic stages of disease remains a high priority due to risks of natural or unnatural outbreaks or epidemics. For the most threatening diseases, diagnostic tests are largely limited to confirmatory use because the time needed for a lab diagnosis test is too long for point-of-care therapeutic intervention. More immediate, actionable test methods are needed for early diagnosis. As one of the most dangerous bacteria that can cause life-threatening diseases in humans, Rickettsial bacteria are an endemic problem in regions across the globe, and attempts to develop a rickettsiae-based bioweapon have also been documented (Azad, 2007). Historically, rickettsiae have been divided into typhus group (TG) and spotted fever group (SFG). The former only has two members, namely *Rickettsia prowazekii* and *Rickettsia typhi*, the agents of epidemic and endemic typhus. On the other hand the SFG has several members including *Rickettsia rickettsii* and *Rickettsia conorii*, the agents of Rocky Mountain spotted fever and other spotted fevers in the Americas and Mediterranean spotted fever, respectively (Nyka, 1950). If prompt and accurate diagnosis is rendered, rickettsial infections can be successfully treated using early and adequate antibiotic therapy. The drug of choice for rickettsial infections is doxycycline, followed by other tetracyclines. Unfortunately, these antibiotics are seldom used as empiric therapy in clinical settings for other undifferentiated febrile illnesses. In fact, some of the antimicrobial agents such as sulfonamides used in the clinical setting can worsen the clinical course of human rickettsioses. (Chapman, 2006; Moody and Chiodini, 2000). The acute phase of all rickettsioses occurs mostly as an undifferentiated febrile illness difficult to distinguish from other more common febrile conditions, leading to the use of ineffective antimicrobial therapy which eventually leads to either a fatal outcome or a more prolonged and severe clinical course

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(Chapman, 2006). Current diagnostics primarily serve as confirmatory tests after treatment choices have been made (Chapman, 2006; Moody and Chiodini, 2000). To be most useful, point-of-care diagnostic assays for rickettsial diseases must be rapid (sample-to-answer time <1 h), highly sensitive, specific and affordable.

A number of technical challenges must be addressed to provide actionable results in time to support treatment decisions. The current gold standard is detection of rising serum antibody titers in paired sera (acute and convalescent samples obtained in at least a 2 weeks interval) by indirect immunofluorescence antibody (IFA) assay. However, this technique has a low clinical sensitivity during the acute phase and therefore appropriate therapeutic selections cannot be made timely. We focus instead on capturing rickettsial organisms using sandwich immunoassays with antibodies raised against *R. conorii* and *R. typhi*. The ultimate goal is to detect circulating rickettsial organisms in human blood during the acute phase of the disease. Existing antigen-capture or direct pathogen test methods are low to moderately sensitive and can be lengthy and costly, and usually require biohazard safety level 3 (BSL-3) facilities and highly trained personnel. Immunohistochemical (IHC) staining of antigens in skin biopsy specimens (Walker, 1995) is highly specific and acceptably sensitive, but requires several hours of labor and an experienced pathologist. Nucleic acid detection by polymerase chain reaction (PCR) or real-time PCR is highly specific and acceptably sensitive for whole blood specimens but requires labor-intensive sample preparation (Eremeeva et al., 2003; Jiang et al., 2003; Stenos et al., 2005). Special shell-vial cell culture assays have been developed for diagnosis of rickettsioses, but 4–15 days of culture in a BSL-3 facility is needed (Birg et al., 1999; La Scola and Raoult, 1996; Quesada et al., 2006).

Here, we describe advancements in microfluidic biosensor technology addressing key requirements for high detection sensitivity and specificity, in a test format poised for rapid, simple and affordable use in point-of-care settings. As a proof of concept, we demonstrate detection of rickettsial organisms in complex biological samples including serum, blood and high backgrounds of non-targeted bacteria. We developed an immuofluorescence-based rickettsial detection assay using an antibody-coated macroporous polyacrylamide monolith (PAM) fabricated within a microfluidic channel to capture and concentrate bacteria from blood samples. Porous polymer monoliths have been explored for wide-ranging uses in microfluidic applications including filtration, pumping and chromatography (Namera et al., 2011; Svec, 2010; Vázquez and Paull, 2010). Acrylamide-based monoliths were previously explored for capillary electrochromatography (Guryča et al., 2007; Palm and Novotny, 1997; Svec, 2010; Xie et al., 1997). Here, the antibody-functionalized PAM filter enables highly effective immunocapture and sensitive detection of rickettsial organisms in a flow-through format (Fig. 1). The PAM exhibits low non-specific background from potentially interfering blood components and non-targeted bacterial species. Performance of the device is demonstrated using serum and blood samples spiked with known quantities of *R. typhi* and *R. conorii*. With spatially resolved fluorescent imaging, individual particles were counted with a detection limit of $\sim 10^2$ cells/ml and linear range up to over 10^5 cells/ml biofluid. A 10 μl sample can be analyzed in about 30 min and larger volumes processed if desired. For acute phase human rickettsioses, the range of rickettsial organisms found in blood circulation is not well defined. A significant technical concern in developing an actionable early diagnostic test is that blood sampling for acute phase diagnostics may require an assay detection limit of as few as ~ 10 bacteria in a relatively large volume of blood (~ 0.1–1 ml). The methods described were developed with the aim of meeting these potential sampling and high sensitivity requirements in addition to affordability, and usability criteria for point of care settings.

2. Material and methods

2.1. Reagents and materials

N,N'-methylenebisacrylamide (bisacrylamide), streptavidin acrylamide, and Alexa Fluor 647 microscale protein labeling kit were from Invitrogen (Calsbad, CA). 40% Acrylamide solution, 3-(trimethoxysilyl)propyl methacrylate (98%), methylcellulose (MC), sodium phosphate, N-methylformamide (NMF), N,N'-diacryloylpiperazine (PDA), tetraethylthiuram disulfide (TED) and 2,2-dimethoxy-2-phenylacetophenone (DMPA), polyethylene glycol (PEG) 8000, Saponin, Atto 488-biotin, and glacial acetic acid were from Sigma (St. Louis, MO). 2,2′-Azobis[2-methyl-*N*-(2-hydroxyethyl) propionamide] photoinitiator (VA-086) was from Wako Chemicals (Richmond, VA). Urethane diacrylate (UDA) and triethyleneglycol dimethacrylate (TEGDMA) were from Sartomer (Exton, PA). PBS buffer (20X) was from Thermo Scientific (Rockford, IL). EZ-link NHS-PEG_4-Biotin was from Thermo Scientific.

Pressure sensitive adhesive (PSA) tapes were from 3 M (St. Paul, MN). Glass cover slips were from Fisher Scientific (Pittsburg, PA). Glass fiber filter membranes were from Sterlitech (Kent, WA).

Heat-inactivated *R. typhi* and *R. conorii* were used in all the assays. To raise polyclonal rabbit anti-*R. typhi* and anti-*R. conorii* antibodies, New Zealand rabbits were immunized with 10^6 rickettsial organisms intravenously every 3 weeks until antibody titers by IFA reached 1:2096. The rabbits were sacrificed humanely according to IACUC protocols and blood was harvested. Serum was separated by a low-speed centrifugation step (4000*g*) and purified using high affinity columns coated with protein G. Heat-killed *Escherichia coli* O157:H7 was purchased from KPL (Gaithersburg, MD). Healthy human serum and human blood was from Innovative Research (Novi, MI).

2.2. Device fabrication

A microfluidic channel layout was fabricated by photolithographic polymerization of urethane diacrylate (UDA) microchannels between two glass coverslips as shown in Fig. 2 and as previously described (Abhyankar and Hatch, 2012). Two glass coverslips (24 × 40 mm, No. 1.5, Fisher Scientific), one with channel via of 1 mm diameter holes cut using laser cutter, and the other without, were first cleaned with methanol and then spaced by two strips of 300 μm-thick double-sided pressure sensitive adhesive (PSA) tape at the edges. For a uniformed acrylate-silane coating on the glass surface to allow subsequent covalent linkage between glass and UDA, a mixture of 2:3:5 (v/v/v) 3-(trimethoxysilyl)propyl methacrylate, glacial acetic acid, and de-ionized water was pipetted into the space between the two glass coverslips and allowed to sit for 30 min inside a sealed container. The coverslips were then rinsed twice with methanol and twice with DI water, and dried with a vacuum.

UDA monomer mixture [48.75% UDA, 48.75% triethyleneglycol dimethacrylate (TEGMDA), 1.5% 2,2-dimethoxy-2-phenylacetophenone (DMPA) and 1% Tetraethylthiuram disulfide (TED)] was prepared with 4 h stirring and mixing enhanced via sonication for 30 min. The UDA monomer solution was pipetted into the 300 μm-thick cavity between the two glass coverslips through the holes on the glass coverslip by manual pipetting. Black electrical tape with laser cut channel design was placed on the cover slip and served as a lithography mask. After illumination by a collimated UV light source centered at 365 nm at 25 mW/cm^2 from a mercury lamp (OAI) (same setting for UV exposure as in the following steps) for 30 s, UDA channel wall was formed at the unmasked region inside between the coverslips. The uncured UDA monomer mixture in the masked regions (where the channels were) was removed by vacuum suction and the channels were