Short Communication

Sequestration of bacteria from whole blood by optimized microfluidic cross-flow filtration for Rapid Antimicrobial Susceptibility Testing

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ABSTRACT

A microfluidic device to separate bacteria from blood cells based on size-exclusion through cross-flow channels was designed and performance tested using fluorescently-labeled, heat-killed *Escherichia coli* spiked into whole blood. The device is easy and cheap to fabricate, and simply and robustly purifies bacteria from large blood cells. Thus the device would be an effective sample-preparation stage within a point-of-care system for rapid testing for antibacterial resistance.

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1. Introduction

Sepsis causes 751,000 hospitalizations and 215,000 deaths in the United States annually, costing $17 billion [1]. Incidence is increasing 13.7% per year, with the number of deaths tripling between 1979 and 2000 [2]. The high mortality and cost of sepsis are partially caused by blood culture testing taking too long (24–72 h) [3], and potentially producing false negatives [4]. The delay leads to the use of a broad cocktail of antibiotics that encourage drug resistance and may still prove ineffective [3]. Microfluidic approaches to sequester pathogens from whole blood could facilitate Rapid Antimicrobial Susceptibility Testing (RAST) and allow specific therapy sooner, with a higher chance of success [5,6]. RAST in combination with antimicrobial stewardship can reduce hospital costs by $20,000 per patient [7].

Current microfluidic approaches to filter blood components exploit chemical [8–10], physical [11–18] and hydrodynamic [19–22] separation mechanisms. Methods based on molecular binding to bacteria miss untargeted pathogens. The simplest and cheapest physical method is size exclusion; it does not require

blood cell lysis, which could interfere with subsequent assays. Hydrodynamic methods, except for margination, require sample dilution. In contrast to other blood separation approaches using cross-flow filtration alone [15,21] or with size-exclusion [18], we aimed to separate bacteria from whole blood without dilution and with minimal clogging, a frequent problem for size-based separation techniques.

In order to sequester bacteria, which cause >95% of sepsis cases [2], from whole blood, microfluidic approaches must be tailored for clinical use. Since the bacteria are in low abundance, the device must process ~1 mL without clogging, and must remove blood cells that could interfere with detection and testing. A portable, point-of-care device should provide a filtered output in minutes without complicated sample preparations or dilutions.

Based on the aforementioned clinical criteria, we rationalized that physical and hydrodynamic separation mechanisms in combination would provide robust sequestration of bacteria from large blood cells. Therefore, the current design is a microfluidic filter ("separator") based on size-exclusion and margination, with continuous, undiluted sample flow. We designed cross-flow filter resistances to minimize clogging by extracting more plasma in the region of the device containing lower hematocrit. Separator performance was evaluated by directly loading with undiluted whole blood spiked with fluorescence-labeled *Escherichia coli*, followed by cell counting. The optimized device removes 97% of RBCs and
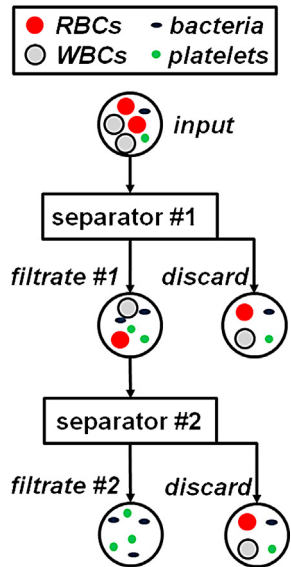
Fig. 1. Block diagram of a microfluidic method to separate bacteria from whole blood. Undiluted, whole blood containing plasma, red blood cells, white blood cells, platelets and bacteria is input to the device. The device separates blood components based upon size. The filtrate receives the smaller fraction, including bacteria. The discard receives most large blood cells. The process can be repeated to further remove blood cells from the filtrate.

retains 30% of bacteria, and so is suitable for sample preparation for point-of-care RAST applications.

2. Materials and methods

2.1. Modeling

The separator was designed to remove half of plasma (~30% of blood volume) from the input, minimize clogging, and sequester bacteria from large blood cells. Fluidic flow was modeled with discrete elements like an electronic circuit. Fluidic pressure was analogous to voltage and fluidic resistance to electrical resistance. Flow was determined by applying Kirchoff's laws, assuming rectangular channels throughout the device, constant viscosity and laminar flow. For a rectangular channel with viscosity μ, length L, width w and height h (where $w > h$), the fluidic resistance is estimated:

$$R_h \approx \frac{(12\mu L)}{wh^3(1 - 0.630h/w)} \tag{1}$$

The separator was modeled as a circuit of independent resistors using Matlab (MathWorks, Inc., Natick, MA). Flow through the last in-series cross-flow channel was half that of the first filter. The flow through each cross-flow channel along the length of the device was set to vary linearly and inversely with the hematocrit in the main channel, which increased steadily from input to output. We reasoned that this flow profile would reduce the chance of clogging. The lengths of the filter channels and the width of the intermediate and collection channel were set to match the resistances from the model using (1). Two columns of cross-flow filters were designed, on either side of the main channel. This maximized filtration speed and reduced clogging.

2.2. Device design and fabrication

A flowchart (Fig. 1) shows the filtration steps. The first and second stage separators were identical (Fig. 2). The separator consists of an inlet for whole blood, leading to two or four main channels in parallel. Cross-flow channels lead on either side to
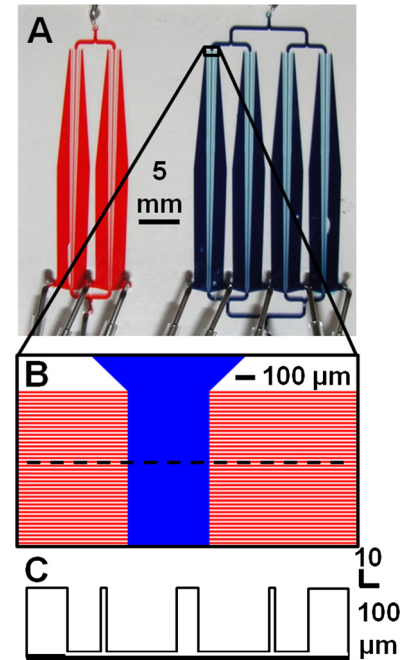

Fig. 2. Separator device design. (A) An *en face* microscope image of two separator devices cast from the same mold, with two (left, red), and four (right, blue) parallel channels to filter blood. (B) An *en face*, and (C) transverse schematic of the filter channel microstructure, depicting main and collection channels (B, blue), and cross-flow filter channel (B, red) dimensions. Scale is indicated. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

two continuous flow collection channels. Filter channel height (1.55 μm) was less than that of RBCs (~2 μm). In the separator, the main channel was 100 μm × 40 μm × 28 mm ($w \times h \times L$); first in-series filter channels were 10 μm × 1.55 μm × 384–45 μm ($w \times h \times L_{FIRST} - L_{LAST}$); first in-series collection channel was 30 μm × 40 μm × 28 mm ($w \times h \times L$); second in-series filter channels were 10 μm × 1.55 μm × 168–179 μm ($w \times h \times L$); and second in-series collection channel was 167–1520 μm × 40 μm × 28 mm ($w_{TOP} - w_{BOTTOM} \times h \times L$). Both feed and collection vials were connected to the device by silastic tubing (0.02 mm ID, VWR International, Radnor, PA) with steel pins.

Negative masks of separator channel microstructures were drawn using AutoCAD (Autodesk, Inc., San Rafael, CA) and printed on transparency masks (CAD ART Inc., Poway, CA). Inverse molds of the required microstructures were fabricated using standard photolithographic steps. Access holes to the inlets and outlets were punched by a 22-gauge punching tool (Technical Innovations Inc., Angleton, TX). The PDMS filter channels were irreversibly bonded to glass slides following 35 s activation with oxygen plasma.

2.3. Sample preparation

Experiments were conducted with human whole blood collected in disodium EDTA-coated vacutainers (Bioreclamation Inc., Charleston, SC, USA). To test the performance of the separator, whole blood was mixed gently with heat-killed, Alexa Fluor 488-conjugated *E. coli* (Life Technologies, Carlsbad, CA) spiked-in at 22 cells/nL. To test the performance of two separators in series, three trials of 1.5, 1.5, and 7.5 mL of whole blood with BioParticles *E. coli* spiked-in was input to the first separators, which had four main channels. The second separators, which had two main channels, received as input the filtrate from the first separators, minus 10 μL, retained for cell counting. The concentrations of BioParticles in stock and RBCs in whole blood were determined by serial dilution and hemacytometer counting.

2.4. Experimental design and analysis

Prior to each experiment, 1% EDTA in PBS was used to fill each device. The separator internal volume was 2.8 μL. Whole blood input to the separator at 750 μL (for single device experiments) or 1.5–7.5 mL (for serial device experiments) were placed into autosampler vials (VWR). To test performance in the serial experiment, filtered output from the first separator was input to a second, identical separator. Flow was driven by 10 psi regulated pressure. The filtration devices were mounted on an inverted microscope (IX-50, Olympus, Japan) equipped with a CCD camera (Marshall Electronics, El Segundo, CA). DP Controller software (Olympus) was used for image acquisition and ImageJ (NIH, Bethesda, MD) for image analysis. Concentrations of RBCs and *E. coli* were determined by hemacytometer counting under brightfield and epifluorescence illumination, respectively. Whole blood input and separator discard were diluted $80\times$, and separator filtrate $16\times$, to accurately count cells. *E. coli* were counted using a $10\times$ objective in a 57 nL region; RBCs were counted using a $40\times$ objective in a 2 nL region. Results are presented for three trials for both experiments, using separate filter devices for each trial. All parameters are listed as mean $\pm$ SD. The retention of *E. coli* was defined as the proportion of *E. coli* retained in filtrate to that of input. The removal of RBCs was defined as the reduction in RBC concentration of the filtrate as a percentage of the input. The bacterial abundance was defined as the ratio of bacteria to RBCs.

3. Results

Whole blood was filtered quickly and without clogging, with higher bacterial purity in the filtrate output. Performance of the separators was tested by three successive runs on independent devices. Three separator devices were loaded each with 750 μL of whole blood spiked with heat-killed, labeled *E. coli*. Pressure-driven filtration was stopped once air entered the input tubes. Complete uptake of the blood occurred in 8 ± 2 min. The time-averaged flow rate was 100 ± 30 μL/min. Filtrate from collection channels weighed 130 ± 20 mg, $17 \pm 2\%$ of the total output. Effluent from the main channel weighed 650 ± 20 mg.

The filters separated *E. coli* from whole blood and removed most RBCs (Fig. 3). The whole blood input spiked with bacteria (Fig. 3A and B) contained 22 ± 2 *E. coli*/nL and 4900 ± 120 RBCs/nL (Fig. 3E and F). Filtrate (Fig. 3C and D) contained 36 ± 2 *E. coli*/nL and 140 ± 40 RBCs/nL (Fig. 3E and F). The retention of *E. coli* was $30 \pm 6\%$, and the removal of RBCs was $97 \pm 2\%$. The discard contained RBCs at 5600 ± 500, demonstrating that most cells did not lyse during filtration.

Two separators in series, with the second receiving filtrate of the first, yield higher bacterial purity than a single device. Three separate trials processed 1.5, 1.6, and 7.5 mL of whole blood. In the third trial, flow through the device stopped after 7.5 mL were processed. The filtrates were loaded immediately into new separators and processed completely, without clogging the devices. Flow rate through the first stage was 70 ± 20 μL/min and through the second stage was 120 ± 50 μL/min. Filtrate from the separators was $11 \pm 6\%$ of output after the first stage, and $44 \pm 20\%$ of output from the second stage, by weight.

Serial filtration with two devices separated *E. coli* from RBCs, removing more RBCs and yielding higher relative abundance of *E. coli* than from a single device. Whereas the input contained 19 ± 1 *E. coli*/nL and 4900 ± 1400 RBCs/nL, the first filtrate contained 28 ± 13 *E. coli*/nL and 170 ± 90 RBCs/nL, and the second filtrate contained 30 ± 7 *E. coli*/nL and 100 ± 20 RBCs/nL. The total (stages $1 + 2$) abundance enrichment of *E. coli* was $8200 \pm 2500\%$, and the total removal of RBCs was $98 \pm 1\%$.
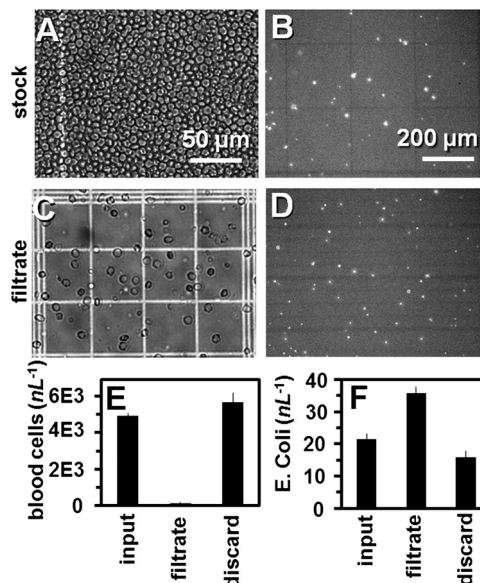

Fig. 3. Sequestration of bacteria from whole blood by a single separator. Representative images from (A, B) input and (C, D) filtrate, $16\times$ diluted, with RBCs visible by (A, C) brightfield and bacteria by (B, D) epifluorescence microscopy. Scale is indicated. Absolute concentrations of (E) large blood cells, and (F) *E. coli* in the device input, filtrate, and discard output. Error bars represent standard deviation.

4. Discussion

Our devices offer several advantages over other approaches. They isolate bacteria without bias because separation is based solely on size differences between bacteria and other blood constituents. Most pathogenic bacteria have $\sim100\times$ less volume than RBCs. In contrast, separation by bacterial ligand capture introduces bias associated with binding specificity. Many microfluidic separation methods require blood dilution, with devices less simple to fabricate and operate. Unlike size-based filtration techniques requiring sheath flow or blood cell lysis, no blood sample processing is required.

Separator performance compares well with other methods [19,20,23]. The bacterial capture efficiency of 30% approaches separation based on surface-modified micropillars (40%) [24,25], magnetic sorting (50%) [23], and inertial focusing (62%) [22]. However, these methods and others require blood dilution [22,24] and/or specific labeling [23]. The removal of RBCs at 98% is comparable to techniques based on inertial focusing (99%), acoustophoresis (99%) [26], ferrohydrodynamics (96%) [19], and cross-filtration + size-exclusion (99.9%) [18]. The last technique is based on similar principles to the device described here, but requires addition of a perfusion buffer to the whole blood input, and would require modification to isolate bacteria instead of leucocytes. In comparison, the device described in this work requires no sample dilution and uses a single, easy-to-load input. Also, channel lengths were optimized to reduce clogging.

The separator is limited by processed volume (~7.5 mL) and bacterial retention (30%). For RAST, sequestration of bacteria from RBCs is more important than retention, because blood cells may interfere with such assays [27–32]. The limitation in processed volume can be trivially overcome by arranging identical devices in parallel. That will also proportionately increase the amount of retained bacteria, although the retention percentage should remain the same. Parallelization will also avoid increasing the processing time, though using devices in series will take proportionately more time. Processing the greater blood volume currently takes >1 h, but

this time can be reduced by adding more devices or processing channels in parallel (Fig. 2A, blue versus red).

5. Conclusions

A continuous-flow, microfluidic fractionation device design was modeled, tested and found to separate heat-killed, fluorescently-labeled *E. coli* from undiluted whole blood, retaining 30% of bacteria and removing 97% (one-stage) or 98% (two-stage) of RBCs, resulting in 8200% higher relative bacterial abundance. Further, the device can process ~100 μL/min, up to ~7.5 mL. Based on the performance described above, this microfluidic filter design would be useful as a preparation step providing purified bacteria for RAST. Small modifications in channel dimensions could switch the device to isolate bacteria, platelets, or leucocytes.

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