Whole Blood Immunoassay Based on Centrifugal Bead Sedimentation

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BACKGROUND: Centrifugal “laboratory on a disk” microfluidics is a promising avenue for developing portable, low-cost, automated immunoassays. However, the necessity of incorporating multiple wash steps results in complicated designs that increase the time and sample/reagent volumes needed to run assays and raises the probability of errors. We present proof of principle for a disk-based microfluidic immunoassay technique that processes blood samples without conventional wash steps.

METHODS: Microfluidic disks were fabricated from layers of patterned, double-sided tape and polymer sheets. Sample was mixed on-disk with assay capture beads and labeling antibodies. Following incubation, the assay beads were physically separated from the blood cells, plasma, and unbound label by centrifugation through a density medium. A signal-laden pellet formed at the periphery of the disk was analyzed to quantify concentration of the target analyte.

RESULTS: To demonstrate this technique, the inflammation biomarkers C-reactive protein and interleukin-6 were measured from spiked mouse plasma and human whole blood samples. On-disk processing (mixing, labeling, and separation) facilitated direct assays on 1-μL samples with a 15-min sample-to-answer time, <100 pmol/L limit of detection, and 10% CV. We also used a unique single-channel multiplexing technique based on the sedimentation rate of different size or density bead populations.

CONCLUSIONS: This portable microfluidic system is a promising method for rapid, inexpensive, and automated detection of multiple analytes directly from a drop of blood in a point-of-care setting.

The advantages of microfluidic technology for diagnostic applications (i.e., assay speed, sample/reagent volumes, automation, small footprint, and cost) have driven widespread development of point-of-care (POC) devices (1–3). A promising system for POC assays is the “laboratory on a disk,” in which fluid flow is driven by centrifugation of a disk functionalized with microfluidic channels. The laboratory on a disk offers the possibility of portable diagnostic instruments the size of a compact disc player that read inexpensive disposable assay disks. Extensive development efforts have yielded multiple types of immunoassay applications, including simple modifications of data compact discs, sophisticated assay platforms, and printed protein arrays (4–11). In addition, there has been substantial progress toward centrifugal systems with self-contained sample processing capabilities for detection of both DNA/RNA and protein (12–17). In a notable recent example, researchers at the Samsung Advanced Institute of Technology developed a fully integrated, portable immunoassay platform capable of directly processing whole blood (11).

However, despite the level of sophistication achieved with these centrifugal immunoassays, there remains room for improvement. Reproducing the entire task sequence in a conventional immunoassay protocol requires several valves and multiple reagent storage compartments. The need for multiple wash steps to remove nonspecifically bound detection agents and proteins is especially taxing on assay design, requiring wash reservoirs, valves, and a voluminous waste reservoir. This complexity results in the need for increased disk space, reducing the potential for parallelization and multiplexing (18). Furthermore, the large number of steps increases the assay run time (typically 30–60 min) and heightens the probability of a single sequence error that invalidates the assay. For applications requiring rapid POC screening with high fidelity from whole blood samples, a fundamentally new technique for conducting immunoassays is needed.

Here we present proof of principle for a simple immunoassay method in which assay beads are labeled.
directly in 1 μL of whole blood and then removed from the sample by centrifugation through a density medium in a single step. This approach greatly simplifies the assay protocol by inherently washing the beads and separating them from blood cells, thereby eliminating multiple sample preparation and rinsing steps. Because the assay beads are initially dispersed in the sample and become concentrated in a compact pellet, the signal is physically intensified compared to a static substrate and can be read from a constant point near the outer edge of the disk. Using a prototype integrated assay disk, we demonstrated rapid (15-min) measurements of interleukin 6 (IL-6) in 1-μL samples of whole blood. We also demonstrated a novel technique for single-channel multiplexing by separating assay beads into layers on the basis of size and/or density through rate-zonal centrifugation.

Methods

SEDIMENTATION FUNDAMENTALS

The method we describe is based on elementary sedimentation principles, as shown in Fig. 1A. A suspension of sandwich assay microspheres, detection antibodies, and analyte-laden sample are layered on top of a medium denser than the bead-sample mixture, but less dense than the individual beads. The beads are functionalized with capture antibodies, and are connected to labeled secondary antibodies via the presence of analyte molecules in the sample during incubation. On centrifugation, the beads sediment through the density medium and become compacted at the bottom of the channel, where they are analyzed. The mean signal from the packed pellet will be proportional to the concentration of analyte in the sample.

The sedimentation rate of spherical particles (Eq. 1) is based on Stoke’s law:

\[
U_s = \frac{2}{9} \frac{(\rho_p - \rho_f)}{\mu} g R^2
\]

where \(U_s\) is the sedimentation velocity, \(\mu\) is the fluid viscosity, \(\Delta \rho\) is the density of the particle, \(\Delta \rho\) is the density of the fluid, \(g\) is acceleration due to gravity or centrifugation, and \(R\) is the particle radius.

Eq. 1 provides powerful design parameters to separate the complexed assay beads from the remaining sample components. Unbound detection antibodies (approximately 7.5-nm diameter) and nontargeted proteins in the sample are efficiently separated from the micron-sized assay beads owing to their small size and slow sedimentation rate (\(U_s\) is proportional to \(R^2\)). Also, during sedimentation any loosely bound proteins or detection antibodies are detached and left behind in the density medium as each individual microsphere is effectively washed with hundreds of times its volume by Stoke’s flow. Finally, the assay beads and density medium can be chosen such that large particles in the sample (i.e., blood cells) sediment at different rates than the beads, thus isolating the assay beads for analysis.

MULTIPLEXING

This simple microsphere sedimentation assay concept can be extended to a unique form of multiplexing within a single channel by using a mixture of sphere populations with distinct size and/or density through rate-zonal centrifugation.
gation the beads will form discreet layers based on their sedimentation rate, and each layer can be read to measure an independent analyte. When based purely on particle size, effective separation is achieved when the smaller diameter particle population from the bottom of the sample region settles only after the larger particles at the top of the sample region have reached this location first (Fig. 1B). From Eq. 1 we can derive a sizing requirement for effectively separating 2 bead populations given a sample height of \(x_1\) and a medium height of \(x_2\), and assuming equivalent bead density and constant effective gravity:

\[
\frac{R_L^2}{R_S^2} \geq 1 + \frac{x_1 \mu_1 \Delta \rho_1}{x_2 \mu_2 \Delta \rho_2}
\]  

(2)

where \(R_L\) is the radius of the larger particles, \(R_S\) is the radius of the smaller particles, \(\mu\) is the viscosity of the sample, \(\mu\) is the viscosity of the medium, \(\Delta\rho_1\) is the density differential between beads and sample fluid, and \(\Delta\rho_2\) is the density differential between beads and the medium. A similar equation can be derived for beads of the same radius but made from materials of differing density.

Eq. 2 shows that, for a worst-case scenario in which the sample and medium have equivalent viscosity and density, and assuming \(x_1 = x_2\), a 41% increase in the radius of the large particle compared to the small particle is necessary for effective separation [realistic media have increased density, reducing the needed increment in radius (for the calculation see the Supplemental Data file in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue5)]. When bead size separation is combined with radial channel multiplexing on a disk, it creates a very powerful multiplexed assay. For instance, if monodisperse beads with a 20% increment in diameter are separable this would allow >15 different bead populations between 0.5 and 10 \(\mu\)m to be read, not including potential separations based on bead density. Current channel dimensions facilitate 20 individual channels per disk, and therefore the theoretical capacity is an intriguing 20 \(\times\) 15 = 300 simultaneous assays per disk.

**REAGENTS**

Anti–C-reactive protein (CRP) and anti–IL-6 antibody pairs were purchased from R&D Systems. The nonbiotinylated antibody from each pair was conjugated to Alexa647 by using labeling kits from Invitrogen. We obtained 1-\(\mu\)m silica and polystyrene beads coated with streptavidin from Bangs Laboratories and 2.8-\(\mu\)m superparamagnetic polystyrene/iron oxide beads coated with streptavidin from Invitrogen. Streptavidin-coated beads were coupled to 150 \(\mu\)mol/L biotinylated anti-body by incubation for 1 h in PBS at room temperature. Following coupling, beads were incubated for a minimum of 3 h at room temperature in PBS containing 1% BSA as a blocking agent, then washed 3 times in PBS. Medium with a density of 1.03 g/cm\(^3\) was formulated from 7%, 10 000-Da molecular weight Dextran (Sigma Aldrich) in PBS containing 0.05% sodium azide, 0.1% BSA, and 0.1% Pluronic F127 (a nonlysing detergent that blocks nonspecific labeling) (19). Whole human blood was obtained by finger prick, anticoagulated by dilution to 40% in PBS containing 2 g/L EDTA, and spiked with varying concentrations of IL-6. Written permission for blood collection and analysis was obtained from all donors according to an institutional review board–approved protocol.

**MICROFLUIDIC FABRICATION**

Microfluidic disks were designed in AutoCAD LT 2000i (Autodesk) and fabricated from layers of double-sided pressure-sensitive adhesive from Fralock. To accommodate on-disk channels of varying depth, disks were constructed from 1 layer of 125-\(\mu\)m polyester (Mylar) film sandwiched between 2 layers of 100-\(\mu\)m adhesive, which in turn were sandwiched between 2 0.5-mm thick copolyester (polyethylene terephthalate glycol) sheets by a process similar to that described elsewhere (Fig. 2, A and B) (17). Stir bars cut from a 275-\(\mu\)m diameter 17–4 PH steel wire (Amazon.com) were placed in partially assembled disks before sealing of the lid to facilitate magnetically assisted mixing (20). Channel patterns were cut from the pressure-sensitive adhesive layers before assembly by using a computer controlled plotter-cutter (Graphtec America). The polyethylene terephthalate glycol disks and the lids containing fluid access holes were laser cut in 18-U batches by Ponoko. Microfluidic disks were assembled by alignment and sealing under manually applied pressure. The resulting disks contained 325-\(\mu\)m chambers connected by 100-\(\mu\)m channels as indicated in Fig. 2B.

On-disk separation columns were preloaded with 1.5-\(\mu\)L density medium before use. We used 1.5 \(\mu\)L of molten octadecane (C\(_{18}\)H\(_{36}\), Sigma Aldrich) to seal in the density medium and to serve as a simple sacrificial phase-change valve as described previously (Fig. 2C) (21, 22). For each assay 1 \(\mu\)L of detector suspension containing PBS with 1% w/v polystyrene IL-6 capture beads, 400 nmol/L detector antibody, 0.1% BSA, and 0.1% Pluronic F127 was prepared and either mixed with sample off-disk or preloaded and stored in the reagent reservoir on-disk (Fig. 2A, iii). All disks were placed in a humidified chamber and refrigerated at 4 °C for up to 1 week until use.
**EXPERIMENTAL PROTOCOL**

**Bead sedimentation immunoassay.** In its simplest incarnation, the design for a bead sedimentation assay is a single valveless microfluidic column oriented radially from the disk center and containing a fluid access port. To initiate this basic assay, a detector suspension containing 1% (w/v) capture beads and 200 nmol/L Alexa 647 conjugated detector antibody was mixed 1:1 with each sample to be analyzed (see Fig. 1A). The sample and detector suspension were mixed and incubated for 10 min off-disk at room temperature. Following incubation, each mixture was introduced into a channel in the microfluidic disk by pipette, and the disk was briefly spun using a battery-powered rotary tool (Dremel Minimite) to layer the mixture on top of the density medium. Finally the disk was spun at 8000–9000 rpm (high setting) for 5 min to pellet the capture beads. Following the centrifugation, bead pellets were imaged with an Olympus microscope employing a 300-W xenon lamp, Cy5 filter set, and 10×0.4 numerical aperture air objective with a consistent exposure time of 20 ms. Image capture settings on the CoolSnap II camera were controlled with ImageJ micromanager software. Measurement of the mean intensity of the pellet image was conducted by using ImageJ. Data were fit with a 4-parameter dose–response curve calculated in GraphPad Prism 5.0 (23). Limit of detection and limit of quantification parameters are included in the ESI.

**Whole blood sample-to-answer assay.** For whole blood processing the disks were operated with a 3-step procedure as illustrated in Fig. 2C. Whole blood samples were initially loaded into the inlets. The disk was spun at pulsed, low (approximately 4000) rpm for 10 min to mix the reagents in the mixing chamber. 3. Temperature is raised slightly to melt the paraffin valve and release the reaction products to the separation column. Assay beads and blood cells are pelleted at the periphery of the disk following a 5-min spin at high (approximately 8000) rpm.

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**Fig. 2. Automated sample-to-answer immunoassay disk.**

(A), Brightfield image of device. (B), On-disk microfluidic channel layout. (C), 3-step blood sample assay protocol: 1. Blood sample and detector suspension (beads and detection antibodies) are loaded into their respective inlet reservoirs. 2. Disk is spun at pulsed, low (approximately 4000) rpm for 10 min to mix the reagents in the mixing chamber. 3. Temperature is raised slightly to melt the paraffin valve and release the reaction products to the separation column. Assay beads and blood cells are pelleted at the periphery of the disk following a 5-min spin at high (approximately 8000) rpm.
beads and sample by way of a piece of steel wire contained in the mixing chamber, which oscillated owing to the balance of centrifugal and magnetic forces. During the final release and pellet step, the disk was spun at 4000–5000 rpm while heat was applied to the disk by using a hand-held heat gun (Chicago Electric) for 4 s to raise the temperature past 28 °C (the melting temperature of octadecane) and release the valve. The disk was then spun continuously at 8000–9000 rpm for 5 min to pellet the IL-6 assay beads and the red blood cells. Because blood cells sediment about 100 times faster than the assay beads, the assay beads formed a distinct layer on top of the cell pellet, eliminating the need for a specialized plasma separation step. A working plasma-separation chamber (Fig. 2B) was nonetheless incorporated into the design to allow flexibility for assays in situations in which the presence of red cells could be problematic.

**Multiplexed assay.** To demonstrate single-channel multiplexing, anti–IL-6 IgGs were coupled to 2.8-μm paramagnetic beads while anti-CRP IgG were coupled to 1-μm polystyrene beads. Theoretically (see Eq. 1), the 2.8-μm superparamagnetic beads (density 1.21 g/cm³) should precipitate through 1.03 g/cm³ medium approximately 70 times faster than 1-μm polystyrene beads (density 1.05 g/cm³) leading to efficient separation of IL-6 and CRP capture beads into layers. Aside from the inclusion of 2 distinct bead populations and 2 distinct labeling antibodies in the detector mix, the single-channel multiplexed assay was run exactly the same as the single analyte assay.

**Results**

**BEAD SEDIMENTATION IMMUNOASSAY**

Raw pellet images used to detect recombinant CRP or IL-6 in spiked mouse plasma are shown in Fig. 3A, and the immunoassay dose–response (calibration) curves in spiked plasma and PBS are shown in Fig. 3B. The dynamic range for IL-6 detection is roughly 3 orders of
magnitude, which is greater than the dynamic range of a typical ELISA (2 orders of magnitude), due in part to the surface area of a pellet of 1-μm beads compared to an equivalent planar surface (approximately 320 times larger). The limits of detection (LODs; measured as the intersection of the calibration curve and 3 SDs over the 0 pmol/L measurement) were determined to be 92 pmol/L and 74 pmol/L for CRP and IL-6 in serum, respectively. The limits of quantification (LOQs; 10 SDs over 0 pmol/L measurement) were calculated as 566 pmol/L and 331 pmol/L for CRP and IL-6, respectively. This detection capability is typical of cytometry-based multiplexed assays, and covers the medically relevant range for serum CRP concentration (24). The mean CVs for all measured concentrations were 17.8% for IL-6 in plasma, 16.9% for IL-6 in PBS, and 13.9% for CRP in plasma, which are within the range of a typical ELISA and below the 20% CV necessary for consistent discrimination of a doubling of analyte concentration (25).

**WHOLE BLOOD SAMPLE-TO-ANSWER ASSAY**

Fig. 4A demonstrates direct sample-to-answer IL-6 quantification from both 1-μL human whole blood and mouse serum samples at a 20% dilution. The brightfield images depict the loading, mixing, valving, and pelleting needed to quantify a target analyte directly from whole blood, along with the assay beads layered upon the blood cell pellet at the periphery of the disk following the final spin. Total assay time for these
runs was 15 min, with all assays conducted simultaneously on a single disk. Fluorescence signal measured from the bead layer on top of the blood cell pellet increased in concert with IL-6 concentration over the tested range (Fig. 4B). For experiments in serum and blood, the magnitude of the signal was comparable to that measured in the more basic assay, as were the extrapolated LODs (63 pmol/L and 168 pmol/L, respectively). In whole blood the mean CV for the measurements was lower than the basic assay (CV of 11.9% compared to 17.8%), and for serum samples the variation was comparable (CV of 16.2%). This improved consistency may result from the steady bead dispersion and incubation time afforded by on-chip mixing. Notably, liquid paraffin generated after the valving operation floated on “top” of both the sample and density medium before solidifying, essentially self-sealing each assay chamber. This paraffin cap may provide a useful additional service in containing the sample and preventing cross-contamination following completion of the assay. The concept of floating paraffin valves has recently also been explored by Abi-Samra et al. (26).

**MULTIPLEXED ASSAY**

The results of the multiplexed experiments are shown in Fig. 5. Fig. 5A shows a brightfield image of the 1-μm
CRP capture beads layered on top of the 2.8-μm CRP capture bead pellet. The larger beads also contain iron oxide, which causes the darker appearance in the brightfield. Fig. 5B shows fluorescent micrographs of layered beads pelleted from samples containing varying concentrations of each analyte. The bead layers are clearly distinct and detect their respective analytes independently of one another in mixed solution. As shown quantitatively in Fig. 5C, the presence of varying concentrations of CRP did not have a detectable effect on the measurement of IL-6 concentration. On the other hand, high concentrations of IL-6 had a modest effect on the measurement of lower concentrations of CRP due to signal crossover from the brighter IL-6 bead pellet (Fig. 5D). This light pollution could potentially be reduced by adding intermediate “spacer” bead pellet (Fig. 5D). Although we detected only 2 analytes, this method of multiplexing is based on the fundamental hydrodynamic properties of the beads, and could theoretically be extended to encompass several analytes.

Discussion

The robust nature of this assay technique is highlighted by its functionality with relatively crude experimental methods. We show that inexpensive and commonly available items such as the rotary tool and a heat gun (such as a handheld hair dryer) can be used to develop centrifugal microfluidic tools with a modest level of complexity and a high level of reproducibility. Because the disk contains all reagents necessary for operation, the instrumentation is light and battery powered, and the mode of detection is ubiquitous within biological laboratories, the current prototype platform achieves a degree of portability comparable with conventional ELISA. However, because this improvised equipment is inadequate for developing a mature POC assay system, there is a need to develop a higher performance device with integrated detection capabilities. For instance, automated sample metering, motor operation, and fluorescence measurement would increase consistency and dramatically reduce time necessary for data acquisition and processing. Because the LOD was largely determined by bead autofluorescence (see Fig. 3, B and C), signal amplification could potentially expand assay sensitivity toward the level of the most diltute protein biomarkers in serum. In particular, long-wavelength emission quantum dots may provide for increased signal intensity and decreased autofluorescence due to a broad spectral shift (10). These and other improvements are well established in previous laboratory on a disk platforms, and may be incorporated into a POC incubation with total sample-to-answer time approaching 10 min or less.

In the meantime, we have demonstrated a simple yet powerful immunoassay approach that outperforms previous laboratory on a disk immunoassays in terms of device complexity, total assay time (>2-fold reduction), and required sample/reagent volumes (>50-fold reduction). The key difference between this approach and previous methods is that instead of mimicking conventional ELISA protocols (i.e., serially flowing samples, detection antibodies, and wash buffer past a fixed capture substrate), our approach removes the solid capture entity (beads) from the sample and detection mixture in just a few steps by exploiting well-understood behavior of fluids and particles of differing density under centrifugation (24). In contrast to previously described cytometry or centrifugal microfluidic bead-based assays, 1 μm or smaller beads are compatible with this assay method, greatly improving the reaction rate and reducing the time required for analysis. Furthermore, we demonstrated the ability to conduct assays directly on microliter volumes of complex samples such as plasma and blood without loss of fidelity. This advanced sample-processing capability is achieved with a minimum of additional valves and reservoirs, making user-friendly and low-cost implementation of the assay well within reach.

The potential applications for fully integrated multiplexed POC molecular analysis solutions are enormous, including doctor’s office testing, patient self-care, remote access to medical care, and national security (1, 27). Because of its inherent ruggedness, speed, analytical power, economy, and diversity of applications, this method has the potential to meet the exacting standards required for POC diagnostics applications.

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