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Digital Microfluidic Magnetic Separation for Particle-Based Immunoassays

Alphonsus H. C. Ng,[†,§] Kihwan Choi,[‡,§] Robert P. Luoma,[∥] John M. Robinson,[⊥] and Aaron R. Wheeler*[,†,‡,§]

[†]Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Toronto, Ontario M5S 3G9, Canada

[‡]Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario M5S 3H6, Canada

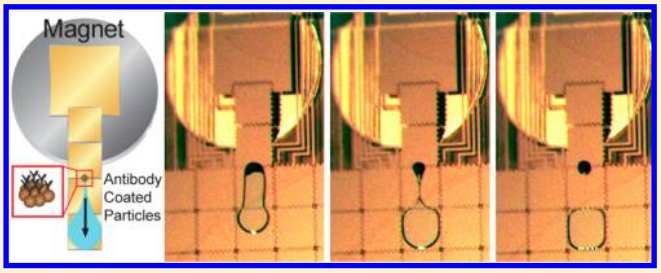
[§]Donnelly Centre for Cellular and Biomolecular Research, 160 College Street, Toronto, Ontario M5S 3E1, Canada

[∥]Abbott Diagnostics, 1921 Hurd Drive, Irving, Texas 75038, United States

[⊥]Abbott Diagnostics, 100 Abbott Park Road, Abbott Park, Illinois 60064, United States

Supporting Information

ABSTRACT: We introduce a new format for particle-based immunoassays relying on digital microfluidics (DMF) and magnetic forces to separate and resuspend antibody-coated paramagnetic particles. In DMF, fluids are electrostatically controlled as discrete droplets (picoliters to microliters) on an array of insulated electrodes. By applying appropriate sequences of potentials to these electrodes, multiple droplets can be manipulated simultaneously and various droplet operations can be achieved using the same device design. This flexibility makes DMF well-suited for applications that require complex, multistep protocols such as immunoassays. Here, we report the first particle-based immunoassay on DMF without the aid of oil carrier fluid to enable droplet movement (i.e., droplets are surrounded by air instead of oil). This new format allowed the realization of a novel on-chip particle separation and resuspension method capable of removing greater than 90% of unbound reagents in one step. Using this technique, we developed methods for noncompetitive and competitive immunoassays, using thyroid stimulating hormone (TSH) and 17β-estradiol (E2) as model analytes, respectively. We show that, compared to conventional methods, the new DMF approach reported here reduced reagent volumes and analysis time by 100-fold and 10-fold, respectively, while retaining a level of analytical performance required for clinical screening. Thus, we propose that the new technique has great potential for eventual use in a fast, low-waste, and inexpensive instrument for the quantitative analysis of proteins and small molecules in low sample volumes.



The immunoassay is a technique that relies on antibody–antigen interactions to quantify relevant analytes in applications such as medical diagnostics, pharmaceutical research, and biological research. The measurement is effected using either a competitive or a noncompetitive assay mode. In competitive mode, exogenous labeled antigens compete with unlabeled antigens from the sample for binding sites on the capture antibodies. In noncompetitive mode, labeled antibodies and capture antibodies bind to the target antigens from the sample at different epitopes, forming an antibody–antigen–antibody complex. In both assay modes, capture antibodies are typically immobilized on a solid support such as on the surface of microtiter well-plates or micrometer-dimension particles (i.e., "microparticles"). Schematic representations of micro-particle-based competitive and noncompetitive immunoassays are shown in Figure 1A.

Since its conception in the 1960s, the immunoassay has grown in demand as the pace of discovery of new disease biomarkers has increased, driving the development of fully automated immunoassay analyzers in the 1980s.[1] These systems represent the current gold standard for immunodiagnostics; however, there are several drawbacks that limit their effectiveness. One drawback is the requirement of large sample volumes, which limits their clinical utility. For example, in disease diagnostics, it would be ideal to assay a single sample of a patient's serum for many different disease markers—this can pose problems when 100–200 μL of sample is required for each test. Another drawback is the cost and time required for each assay. Because these systems rely on robotic instrumentation for fluid handling, they are expensive to maintain and operate, restricting their use to wealthy laboratories and centralized facilities. Analysis can take up to several hours because analyte molecules must diffuse across long distances before they encounter antibodies on the solid phase. A

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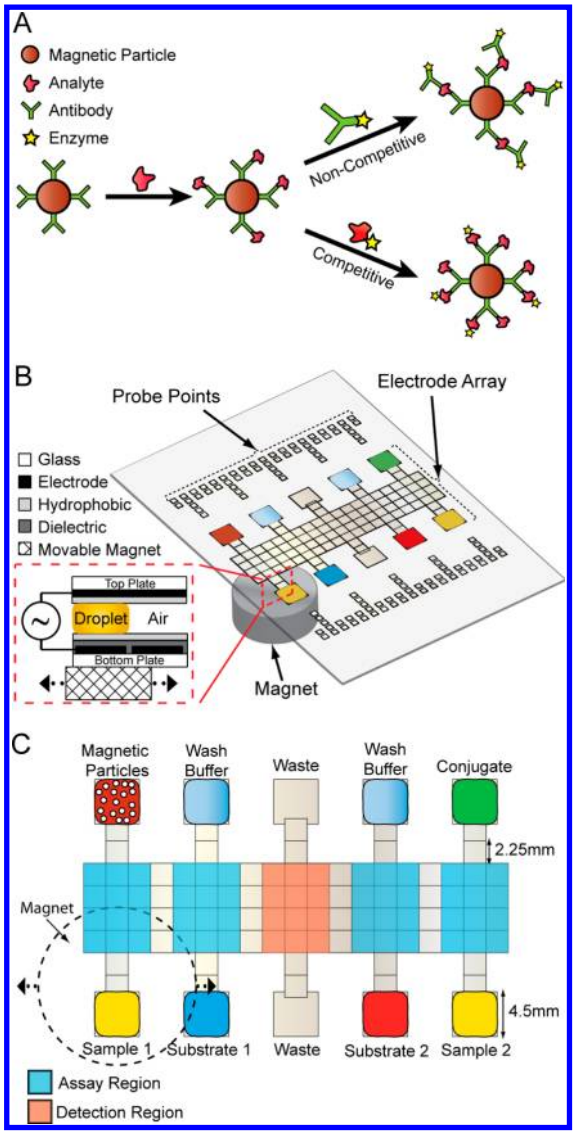

Figure 1. Immunoassays and digital microfluidics. (A) Scheme of noncompetitive and competitive immunoassays using magnetic particles. (B) Three-dimensional schematic and side view of a DMF device, which features a moveable magnet underneath the device for particle immobilization. (C) Top view of a DMF device, showing the position of the magnet, 10 reservoirs to accommodate reagents, 96 actuation electrodes to carry out the immunoassay protocol, and dedicated regions for assay and detection steps.

potential solution to these drawbacks is the miniaturization of immunoassays using microfluidics.[2] The smaller dimensions of microfluidics can reduce diffusion times and reagent consumption, resulting in faster analysis and lower cost per assay. In addition, fluid handling in microfluidics can be automated with simple, compact instrumentation, reducing the size and operating cost of test equipment. In the past decade, significant effort has been focused on developing microfluidic immunoassays with varying configurations and fluid handling schemes. Among the wide range of assay configurations, the use of microparticles as solid support is attractive as this approach offers a dramatic increase in the surface-area-to-volume ratio and serves as a simple mechanism to reproducibly deliver antibodies into a microfluidic device. Paramagnetic particles that respond to external magnetic fields are particularly appealing as they can be easily immobilized without the need for microfabricated structures for physical retention in the device.[3] The magnetic forces exerted on paramagnetic particles can also enhance mixing,[4] focus particles for immunoagglutination assays,[5] and serve as a detection mechanism for immunoassays.[6] While most fluid handling schemes in microfluidic immunoassays rely on continuous fluid flow[7] or droplets[8] within networks of enclosed micrometer-dimension channels (i.e., microchannels), an alternative droplet-based fluid handling scheme called digital microfluidics (DMF) is growing in popularity.[9−11] In DMF, fluids are electrostatically controlled as discrete droplets (picoliters to microliters) on an array of insulated electrodes. By applying a series of potentials to these electrodes, droplets can be made to merge, mix, split, and dispense from reservoirs.[12] Since droplets are manipulated on a generic array-geometry, multiple droplets can be controlled simultaneously and droplet operations can be reprogrammed without changing device design.[13] This flexibility makes DMF suitable for applications that require complex, multistep protocols such as immunoassays.[14,15]

To date, two DMF methods for magnetic particle-based immunoassays have been reported. In the first approach, Sista et al.[16,17] demonstrated noncompetitive immunoassays for insulin, interleukin-6, and troponin I using 1.05 μm diameter paramagnetic particles. In these methods, a solution containing a mixture of magnetic particles, labeled antibodies, and blocking proteins was prepared off-chip and then dispensed, merged and mixed with a droplet of analyte on-chip to form antibody−antigen complexes. Subsequently, a "serial dilution" method was used to wash unbound reagents from magnetic particles. To prevent nonspecific adsorption of proteins onto the device surface, the device was filled with an immiscible carrier fluid (1.5 cSt silicone oil), forming a barrier between the droplet and the surface. In the second approach, Vergauwe et al.[18] explored the use of 15 nm diameter paramagnetic particles for noncompetitive immunoassays for IgE. The authors also employed the serial dilution method (as above); but rather than completely filling the device with silicone oil, each of the aqueous sample and reagent droplets were encapsulated by thin oil shells.

The microparticle immunoassay techniques reported previously[16−18] represent important milestones, but they suffer from some limitations. First, both techniques describe noncompetitive immunoassays only; competitive immunoassays (which are important for many clinical applications) have never been reported in digital microfluidic systems. Second, both techniques require significant off-chip sample and reagent preparation. Third, both approaches make use of an oil carrier fluid. Oil is useful for DMF systems in that it (a) enables droplet actuation at lower voltages, (b) reduces the amount of surface adsorption, and (c) significantly reduces the droplet evaporation rate.[19] But there are also several disadvantages of using oil, including (1) proteins in sample and reagent droplets may adsorb to the water−oil interface,[20] (2) oil is problematic for integration with other on-chip functions including cell culture,[21] surface-based assays,[22] and liquid−liquid/solid−liquid extraction,[23] and (3) specialized device packaging is required to prevent leaks.[24]

Here, we report a new format for DMF magnetic particle-based immunoassays, with several improvements relative to the techniques reported previously. First, we report the first competitive immunoassay implemented by digital microfluidics, for 17β-estradiol (E2). [To illustrate the versatility of the technique, we also developed a noncompetitive assay for

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thyroid stimulating hormone (TSH).] Second, the new method allows for each of the required assay reagents to be injected individually (with no premixing), such that the entire assay can be performed on-chip. Third (and perhaps most importantly), the method reported here was designed to not require the use of an oil filler fluid; i.e., the droplets in this method are surrounded by air. Although oil-free DMF systems have been used to manipulate magnetic particles for other applications,[25,26] this work represents the first oil-free DMF method for particle-based immunoassays. This change in format allowed the realization of a novel on-chip particle separation method, representing a significant improvement to the particle separation methods used previously.[16−18] We propose that the new technique has great potential for use in a fast, low-waste, and inexpensive instrument for the quantitative analysis of proteins and small molecules in low sample volumes.

EXPERIMENTAL SECTION

Reagents and Materials. Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON). Deionized (DI) water had a resistivity of 18 MΩ·cm at 25 °C. Pluronic L64 (BASF Corp., Germany) was generously donated by Brenntag Canada (Toronto, ON). E2 and TSH well-plate ELISA (enzyme-linked immunosorbent assay) kits were purchased from Calbiotech (Spring Valley, CA).

Magnet Movement. A magnet manifold was formed by removing selected wells from a Stripwell microplate. A 5/8 in. diameter × 1/4 in. thick, N48 grade, 15.3 lb pull force neodymium magnet (Emovendo Magnets & Elements, Petersburg, WV) was positioned in the manifold; the missing wells form a groove that delineates allowable magnet positions. In experiments, a DMF device was positioned on top of the manifold (Figure 1, parts B and C). Magnet position was controlled manually using a metal clip, which, when moved, caused the magnet to move to the desired position. Calculations of the magnetic forces applied to the device in this system are included in the Supporting Information.

Magnetic Particle Washing Protocol. A wash-test assay was developed to evaluate the washing efficiency of the serial dilution[16,17] and supernatant separation methods. A wash-test suspension was prepared by resuspending anti-β TSH particles in TSH conjugate solution at ∼3.0 × 10^8 particles/mL. For serial dilution washing, DMF was used to merge and mix a droplet of wash-test suspension with a droplet of wash buffer. Next, the magnet was positioned such that the particles were immobilized to one side of the pooled droplet, the droplet was split into two daughter droplets, and the droplet not containing particles was moved to waste. After the magnet was removed, the droplet containing the particles was mixed to resuspend the particles in solution. For supernatant separation washing, particles in a droplet of wash-test suspension were first immobilized by the magnet and DMF was used to actuate the supernatant droplet away from the magnet to waste, leaving the particles immobilized on the device surface. Next, the magnet was removed, the particles were reconstituted in a droplet of wash buffer, and the droplet was mixed to resuspend the particles in solution. For both techniques, the washing procedure was repeated four times; after each wash step, the supernatant waste droplet was collected by removing the top plate. Using a pipet, the waste droplet was diluted in 50 μL of wash buffer and transferred to a well in a transparent 96-well plate. To this mixture, 100 μL of TMB reagent from a Calbiotech TSH ELISA kit was added to each well. After incubating for 3 min at room temperature, the reaction was stopped by adding 50 μL of stop solution from the ELISA kit. Within 15 min, the absorbance at 450 nm was measured with a Sunrise microplate reader (Tecan, Durham, NC) in "Normal" read mode. Three replicates were evaluated for each condition.

On-Chip Immunoassay Protocol. Using the supernatant separation method (described above) for particle separation and resuspension, an eight-step protocol was developed to implement on-chip immunoassays. (1) A droplet containing paramagnetic particles was dispensed from a reservoir and separated from the diluent. (2) Three droplets containing sample were dispensed and delivered to the immobilized particles for resuspension and incubation for 6 min. (3) The particles were washed four times in wash buffer and separated from the supernatant. (4) One droplet of conjugate solution was dispensed and delivered to the immobilized particles for resuspension and incubation for 2 min. (5) The particles were washed four times in wash buffer, two times in H_2O_2, and separated from the supernatant. (6) One droplet of H_2O_2 was dispensed and delivered to the immobilized particles for resuspension and incubation for 2 min. (7) The droplet of H_2O_2/particle suspension was merged and mixed with one droplet of luminol/enhancer solution. (8) The pooled droplet was incubated for 1 min, and the chemiluminescence was detected with a well-plate reader (Pherastar, BMG Labtech, Cary, NC). To prepare for measurement, the device was affixed with tape to an empty 96-well Stripwell plate so that each droplet was aligned to a well. The device/plate assembly was inserted into the plate reader and evaluated with focal height = 8.6 mm, gain = 3600, using the LUM module.

As shown in Figure 1C, each DMF device had four separate assay regions (for four individual assays) and a shared detection region. The assay regions were used for steps 1−5 of the protocol. After the final rinse, the particles were delivered to the detection region for steps 6−8 of the protocol. Between measurements, the detection region was rinsed by moving droplets of H_2O_2 over the electrodes. The device was disposed after four assays.

DMF Immunoassays. Using the protocol above, a calibration curve was generated on-chip from standard solutions of TSH (0, 0.4, 1, 4, and 20 μIU/mL) or E2 (0, 10, 50, 500, and 1000 pg/mL). Four measurements at each concentration were averaged and fitted to a linear equation (TSH) or a four-parameter logistic equation[27] (E2). The limit of detection (LOD) was the concentration corresponding to the position on the curve of the average signal generated from blank measurements plus (for TSH) or minus (for E2) three times the standard deviation of the blank measurements.

Well-Plate Experiments. Calbiotech ELISA kits (with antibodies immobilized on the surface of transparent 96-well plates) for TSH and E2 were used following manufacturer's instructions. Briefly, the absorbances at 450 nm were measured using a Sunrise microplate reader in "Normal" read mode, and calibration curves were generated and LODs determined as above.

RESULTS AND DISCUSSION

Droplet Movement and Biofouling. In digital microfluidics, droplet movement is impeded by drag forces arising from differential surface energy. Many of the reagents used for immunoassays, including protein solutions and protein-coated particles, are susceptible to nonspecific adsorption (or

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"fouling"), which locally increases the device surface energy, impeding the movement of aqueous droplets.[20]

To alleviate problems of protein fouling, most researchers use either (a) silicone oil filler fluid[28] or (b) Pluronic additives in reagent droplets.[29] Although silicone oil filler fluid is useful for many applications, it is not a universal solution and has some disadvantages (as described in the introduction). The latter technique, employing Pluronic additives, obviates the need for oil. In this work, Pluronic L64[30] was included at 0.05% (v/v) in all reagent and samples solutions to facilitate robust droplet operations without oil (this addition was found not to interfere with specific binding in well-plate or DMF immunoassays). After the addition of L64, most assay reagents were compatible with DMF actuation without further dilution or modification. The exceptions were the ARCHITECT particle diluent, conjugate diluent, and wash buffer, which did not move well even after the addition of L64. We speculate that there are some cross-effects between the (added) L64 and the (native) surfactants in these solutions. This problem was solved by developing custom DMF-compatible particle diluent, conjugate diluent, and wash buffer containing L64 as the only surfactant. All reagent formulations are detailed in the Supporting Information.

Development of On-Chip Washing and Solvent Exchange Protocol. A key requirement in heterogeneous immunoassays is the ability to remove unbound reagents (e.g., antibodies or antigens) from the surface of the solid support. All of the magnetic particle-based immunoassays implemented in DMF reported previously[16−18] have used a "serial dilution" method to wash unbound reagents from particles as depicted in Figure 2A. First, the particles are immobilized by an external magnet. Second, the droplet containing the particle suspension is diluted by merging with a droplet of wash buffer. Third, DMF is used to split the pooled droplet and remove the excess solution from the particles. This process is repeated until sufficient dilution is achieved to minimize the background noise of the assay. This washing technique is inherently inefficient because (a) unbound reagents are slowly diluted over many washes and (b) particles are immobilized by the magnet throughout the wash process, which can trap unbound material between particles.[16]

We hypothesized that we could improve upon the performance of the serial dilution method by using an alternative approach that allows for the near-complete separation of the supernatant solution phase from the particle solid phase in one step (similar methods were recently reported for different applications in digital microfluidics[31] and other droplet/microfluidic formats[32,33]). We proposed that this "supernatant separation" method (Figure 2B) would be capable of much more efficient isolation of particles from unbound reagents. In this method, a magnet causes particles to focus into a pellet and immobilize on the device surface, such that the pellet remains behind when the supernatant droplet is actuated away. After separation from the supernatant, the immobilized particles can be resuspended by removing the external magnet, passing a fresh droplet over the pellet, and shuttling the droplet in a circular motion across four electrodes.

We developed a wash-test assay to evaluate the efficiency of both the supernatant separation and serial dilution methods. In each assay, a droplet containing anti-β TSH particles in anti-TSH−HRP solution was washed by one of the two methods, and the resulting waste droplet was interrogated for peroxidase activity. This process (wash and measure) was repeated four times. To ensure an unbiased comparison, identical wash buffer volumes, particle suspension volumes, and particle densities

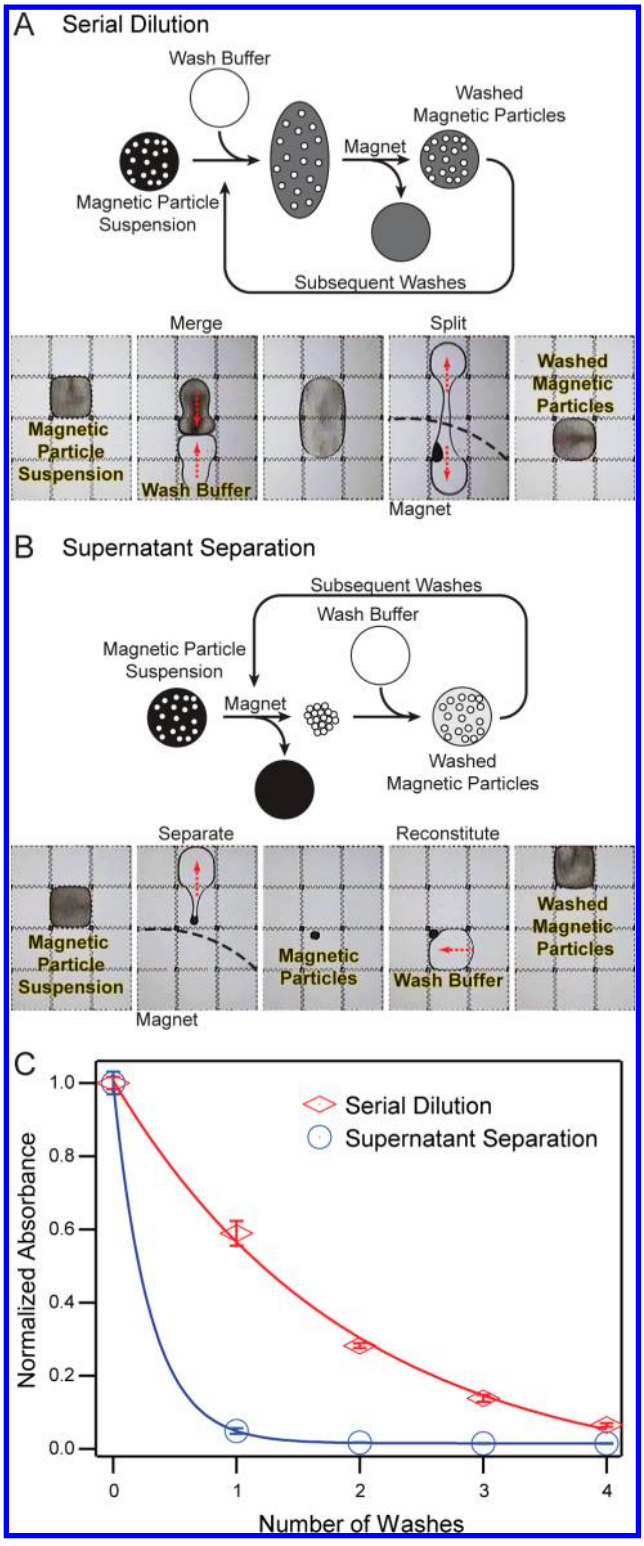

Figure 2. Comparison of magnetic particle washing protocols. (A) Scheme (top) and video sequence (bottom) of serial dilution washing protocol. (B) Scheme (top) and video sequence (bottom) of supernatant separation washing protocol. (C) Comparison of the efficiency of serial dilution (red diamonds) and supernatant separation (blue circles) methods by measuring enzyme activity in the supernatant after each wash step. Error bars are ±1 SD from three replicates.

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were used for both methods (within the range of standard error). To equalize the conditions further, a modified version of the serial dilution method was used (removing the magnet during mixing and particle resuspension), which differs from the previously reported serial dilution methods[16−18] in which particles are immobilized by the magnet throughout the wash process. Finally, in both methods evaluated here, the initial solution and wash solution had the same volume (i.e., 1:1 wash/initial); in previous work,[17] larger ratios (e.g., 5:1) were found to increase the washing efficiency.

As shown in Figure 2C, the supernatant separation method is much more efficient than the serial dilution method. If we define 98% reduction in HRP activity relative to the activity in the initial droplet as an arbitrary benchmark for minimizing background signal of the assay, the supernatant separation method achieves this target within two washes, while the serial solution requires at least six washes. In fact, this analysis may understate the advantage of the supernatant separation method, as our modified version of the serial dilution method is apparently more efficient than the previously reported methods, in which 10−18 washes[16] were required for a 1:1 wash and five washes[17] were required for a 5:1 wash. This is likely because our modified serial dilution method (in which the magnet is removed during mixing and particle resuspension) is less susceptible to the trapping of unbound enzymes between particles.

There are three requirements for successful implementation of the supernatant separation method: (1) sufficient magnetic force, (2) adjustable magnetic force, and (3) stable particle suspension. For the first requirement, the magnetic force immobilizing the particles must be stronger than the interfacial force pulling the particles into the droplet. For a single micrometer-dimension paramagnetic particle in the presence of a magnet, the interfacial force is 2−3 orders of magnitude higher than a typical magnetic force that might be applied in such a system.[24] However, because the interfacial force is proportional to the particle radius and the magnetic force is proportional to the cube of the particle radius, the magnetic force is dominant for particles larger than a critical size. A pellet comprising many microparticles can satisfy this requirement, and in the system used here, pelleting and separation was observed to be possible for droplets containing at least ∼3.0×10^8 particles/mL. From numerical simulations described in the Supporting Information, we estimate that this configuration corresponds to a magnetic force of ∼470 μN (Supporting Information Figure S1C). For the second requirement, after the particles are separated from the origin droplet, the magnetic field strength should be adjustable to permit particle resuspension in a new droplet. In the system described here, this was accomplished by moving the magnet (horizontally) away from the device. For the final requirement, the particles should not dry or irreversibly aggregate during the time spent as a pellet on the surface. In the system reported here, when the origin droplet is removed, we observe that a small amount of liquid (approximated by imaging to be ∼5% of the origin droplet volume) is retained by the hydrophilic particles. To minimize the potential for particle aggregation or drying, the particles are then resuspended immediately in a new droplet. Other factors that may determine particle stability include diluent composition and surface chemistry of particles. Finally, we propose that some combination of these three requirements makes oil-immersed digital microfluidic devices incompatible with the supernatant separation method, but more work is required to evaluate this hypothesis.

In the final analysis, the choice between the serial dilution method and the supernatant separation method likely depends on the application. The former method is less efficient, but can be implemented with a static magnetic field, making it attractive for instruments with low complexity and small footprint. The latter method is more efficient, but requires an adjustable magnetic field, making it better suited to larger, more complex instruments. In the work reported here, the supernatant separation method was used, and we propose that it will be useful tool for a variety of circumstances in which a movable permanent magnet (or an electromagnet with adjustable forces[34]) is available.

Immunoassay Protocol Development. To demonstrate that DMF immunoassays can be adapted for a wide range of analytes, we developed a noncompetitive immunoassay (the type of assay used in all previous DMF methods) and a competitive immunoassay (new for DMF) using TSH and E2 as model analytes, respectively. The TSH immunoassay is used to evaluate thyroid function and diagnose thyroid disease and, thus, is one of the most commonly performed immunoassays in clinical laboratories. The normal serum range of TSH is 0.4−3.0 μIU/mL, although this is frequently revised.[35,36] Estradiol (E2), a small molecule steroid (272.38 Da), is the most potent natural estrogen in mammals. E2 serum levels for males, premenopausal females, and postmenopausal females are 10−50, 30−400, and less than 30 pg/mL, respectively (reported ranges vary between different laboratories[37]).

A number of competing requirements were evaluated in developing the methods reported here. In a traditional immunoassay, reagent volume, incubation duration, and reagent composition should be optimized to achieve the greatest signal-to-noise ratio while minimizing assay time and reagent use. But for the methods reported here, these parameters were also observed to affect the reliability of droplet manipulations on DMF; unreliable droplet movement should be avoided, as it leads to increased assay variability.[38] Balancing these requirements led us to select a sample volume of three unit droplets (800 nL × 3 = 2.4 μL): this provided significant signal improvement over one sample droplet, yet the volume was still small enough to be easily manipulated by DMF (volumes much larger than a unit droplet are more difficult to control reliably). Likewise, for each of the other reagents (wash buffer, particle suspensions, conjugates, and substrates), we used one unit droplet (800 nL) to minimize reagent consumption and to ensure reliability of merged-droplet manipulation. In preliminary experiments, a sample incubation of 6 min was sufficient to saturate the analyte on particle surfaces (data not shown). For this incubation time, sample droplets did not experience significant volume decrease due to evaporation. In fact, we observed that at least 30 min is required for a 1 μL wash buffer droplet to lose 5% of its original volume (Supporting Information Figure S2). For chemiluminescent detection, the reagent manufacturer recommends mixing equal parts H_2O_2 solution and luminol/enhancer solution prior to the assay; however, we found that the signal generated following this procedure exhibited high interassay variability (data not shown). Therefore, we developed an alternative procedure comprising two H_2O_2 wash steps to remove leftover wash buffer solution and an on-chip mix step of the H_2O_2 and luminol/enhancer solutions immediately prior to use. For the conditions evaluated, the chemiluminescence intensity was

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observed to saturate after 1 min—this was chosen as the measurement end point.

Among all of the parameters, we found that the reagent composition is the most critical in determining the performance of the assay—we highlight two examples here: (1) In E2 immunoassays, the concentration of conjugated antigen (E2–HRP) is essential in determining the dynamic range and sensitivity of the assay. In particular, in initial studies using high E2–HRP concentrations, it was impossible to distinguish between different E2 sample concentrations (e.g., 50 and 500 pg/mL have similar signal). The performance of the assay greatly improved when we determined the optimal (lower) concentration of 1 μg/mL. (2) TSH immunoassays are very sensitive to the nonspecific adsorption of conjugated antibodies on the surface of magnetic particles. In initial TSH immunoassay experiments, the high background noise prevented consistent differentiation between 0 and 0.4 μIU/mL analyte. Initially, we tried adding protein, serum, or surfactant components to reduce nonspecific adsorption, but several of these options compromised droplet movement reliability. To alleviate this problem, we developed a technique to block the TSH microparticles using nonfat dried milk[39] prior to the assay (as described in the Supporting Information), which significantly reduced the background noise without affecting droplet actuation.

Optimized Immunoassay. Figure 3A illustrates the optimized immunoassay procedure for both (competitive) E2 and (noncompetitive) TSH immunossays by DMF. As detailed in the Experimental Section, both assays used identical droplet actuation sequences and washing protocols, but with different microparticles and conjugate solutions. The highlights of this procedure are represented in six frames from a movie, shown in Figure 3B. First, magnetic particles were separated from solution. Second, the particles were resuspended in three droplets of sample and incubated for 6 min. Third, after four wash steps, the particles were resuspended in one droplet of conjugate (containing HRP-labeled antibody or analyte) and incubated for 2 min. Fourth, after four wash steps, the particles were reconstituted in one droplet of H_2O_2 and incubated for 2 min. Fifth, one droplet of luminol/enhancer solution was merged with the H_2O_2 to activate enzymatically driven chemiluminescence. Finally, sixth, chemiluminescence was measured in a well-plate reader with detection zone aligned to 96-well plate format.

We carried out this protocol for various concentrations of standards to generate calibration curves for E2 and TSH as shown in Figure 4. Each curve has a dynamic range of approximately 2 orders of magnitude, and the error bars represent the standard deviation between four measurements performed on-chip. The E2 and TSH data have coefficients of variation (CVs) ranging from 9% to 21% and 5% to 14%, respectively. As listed in Table 1, these CVs are similar (but slightly elevated) relative to those observed for comparable assays carried out in multiwell plate format. We note that the DMF assays reported here were implemented semimanually by applying voltage sequences to the device using hand-held voltage probes, and we attribute some of the variability to differences in droplet movement sequences and timing. We propose that this variability will be reduced upon implementation of automated fluid handling with feedback control (as has been observed previously[38,40]).

The most important characteristic of immunoassays is the LOD. As listed in Table 1, the DMF assays had ~2–3× lower

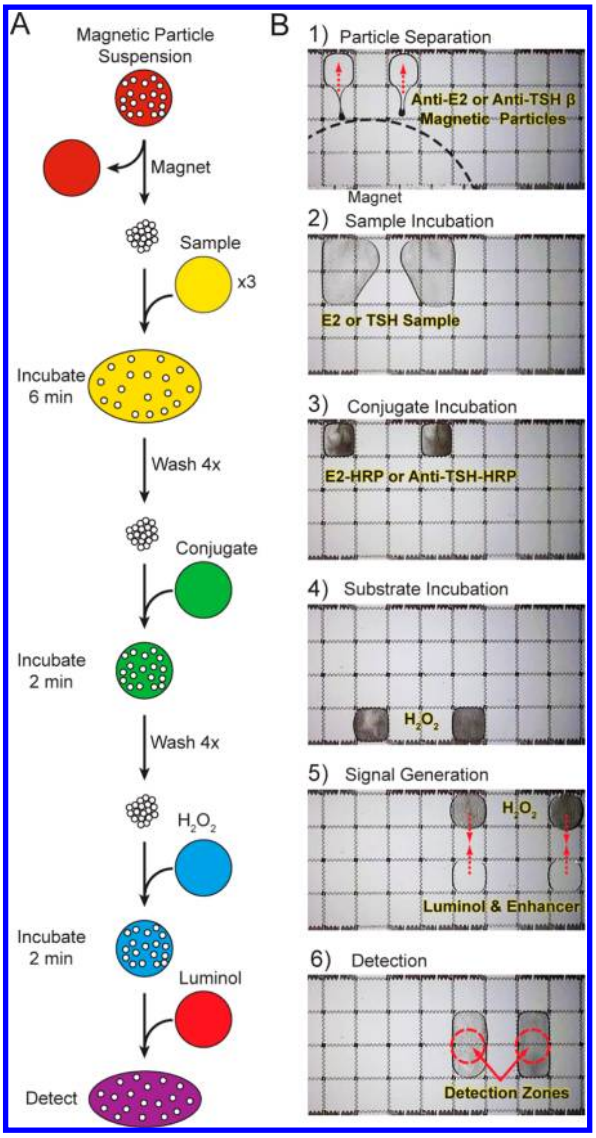

Figure 3. Digital microfluidic immunoassay procedure. (A) Scheme of droplet-based on-chip immunoassay. The E2 and TSH immunoassays use the same droplet actuation sequences and supernatant separation technique for particle washing. (B) Video sequence of on-chip immunoassay procedure, showing two assays in parallel: (1) magnetic particles are separated; (2) sample reconstituted the particles and incubated; (3) after wash steps, droplet of conjugate reconstituted the particles and incubated; (4) after wash steps, droplet of H_2O_2 reconstituted the particles and incubated; (5) droplet of luminol/enhancer solution merged to activate enzymatic reaction; (6) chemiluminescence is measured.

LODs relative to well-plate assays when calculated in terms of absolute amount but were ~3–10× higher when calculated in terms of concentration. The LODs of the DMF system are suitable for a wide range of applications, including the prognosis and diagnosis of hypothyroidism[36] (TSH > 3.0 μIU/mL) or ovarian cancer[41,42] (E2 > 20 pg/mL, depending on patient age). But for some applications, it may be useful to develop modified assays with lower LODs; this might be possible by increasing the amount of sample (e.g., analyte in multiple droplets of sample may be concentrated on the particles through several solvent exchanges until sufficient sensitivity is achieved). In addition, the use of a dedicated detector may help improve the assay sensitivity and variance—

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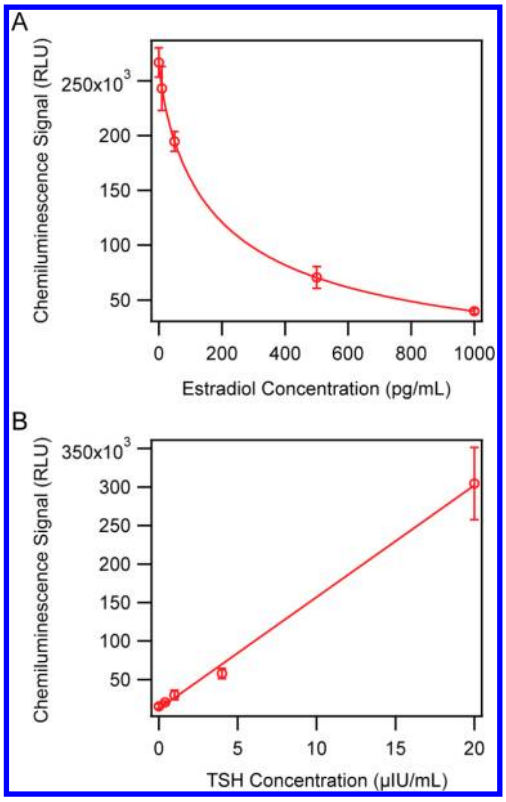

Figure 4. Calibration curves for immunoassays implemented using digital microfluidics. (A) 17β-estradiol (E2) competitive immunoassay. (B) Thyroid stimulating hormone (TSH) noncompetitive immunoassay. Error bars are ±1 SD from four replicates.

Table 1. Comparison of Well-Plate ELISA Kit and DMF Immunoassays

immunoassay system	well-plate ELISA kit		DMF	
analyte	TSH	E2	TSH	E2
coefficient of variability	2–13%	4–12%	9–21%	5–14%
LOD (absolute)	3.0 nIU	152 fg	2.0 nIU	50 fg
LOD (concentration)	0.06 μIU/mL	6.1 pg/mL	0.83 μIU/mL	21 pg/mL
sample volume	50 μL	25 μL	2.4 μL	2.4 μL
sample incubation time	60 min	60 min	6 min	6 min

the calibration curves used here were obtained using a multipurpose (fluorescent, luminescent, absorbance) well-plate reader that is used regularly by a number of different users. Note that LODs and CVs were not reported for the DMF microparticle-based immunoassays reported previously.[16−18]

There are two salient advantages of DMF for immunoassays: reduced consumption of reagent volumes and faster analysis time. As context for the former advantage (reagent consumption), a typical well-plate ELISA assay requires 50 μL of sample, 100 μL of conjugate, 900 μL of wash buffer, and 150 μL of substrate. In contrast, DMF immunoassay requires 2.4 μL of sample, 0.80 μL of conjugate, 6.4 μL of wash buffer, and 1.6 μL of substrate. This represents a 100-fold reduction in reagent consumption in the DMF method, from 1.2 mL to 11.2 μL. For the latter advantage (analysis time), sample incubation in well-plate ELISA kits requires 60 min, and particle-based immunoassay in ARCHITECT immunoanalyzers requires 18 min. In contrast, the DMF immunoassays only require 6 min of sample incubation time. This advantage is directly related to the reduction of reagent volumes—the same number of particles is packed into a smaller volume, increasing surface area-to-volume ratio and reaction kinetics.

CONCLUSIONS

We have developed the first particle-based immunoassay on DMF without the aid of oil carrier fluid. This new format allowed the realization of a novel on-chip magnetic particle separation method capable of removing greater than 90% of unbound reagents in one step. Using this technique, we successfully developed the first DMF-driven competitive immunoassay (for E2), as well as a noncompetitive immunoassay (for TSH). Compared to conventional techniques, this method reduced reagent volumes and analysis time by 100-fold and 10-fold, respectively, while retaining a level of analytical performance required for clinical screening. We propose that the new technique has great potential for eventual use in a fast, low-waste, and inexpensive instrument for the quantitative analysis of proteins and small molecules in low sample volumes.

ASSOCIATED CONTENT

Supporting Information

Details relating to device fabrication and operation, the reagent formulations used on-chip, microparticle preparation, estimation of magnetic force, and measurement of droplet evaporation rate. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: aaron.wheeler@utoronto.ca. Phone: (416) 946 3864. Fax: (416) 946 3865.

Notes

The authors declare no competing financial interest.

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