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Digital Microfluidics for Immunoprecipitation

Brendon Seale,[†] Charis Lam,[†] Darius G. Rackus,[†,‡] M. Dean Chamberlain,[†,‡] Chang Liu,[§] and Aaron R. Wheeler*[,†,‡,∥]

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

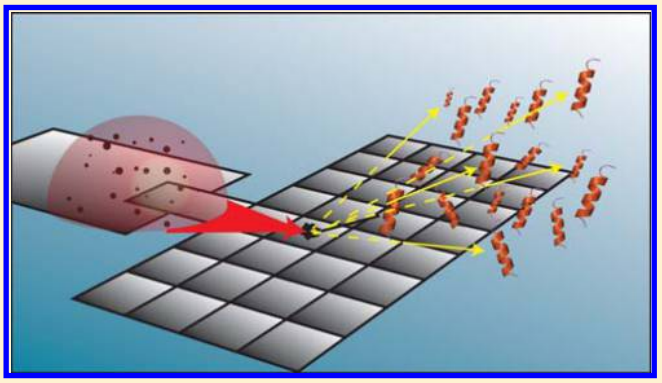
[‡]Donnelly Centre for Cellular and Biomolecular Research, 160 College Street, Toronto, Ontario M5S 3E1, Canada

[§]SCIEX, 71 Four Valley Drive, Concord, Ontario L4K 4V8, Canada

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

Supporting Information

ABSTRACT: Immunoprecipitation (IP) is a common method for isolating a targeted protein from a complex sample such as blood, serum, or cell lysate. In particular, IP is often used as the primary means of target purification for the analysis by mass spectrometry of novel biologically derived pharmaceuticals, with particular utility for the identification of molecules bound to a protein target. Unfortunately, IP is a labor-intensive technique, is difficult to perform in parallel, and has limited options for automation. Furthermore, the technique is typically limited to large sample volumes, making the application of IP cleanup to precious samples nearly impossible. In recognition of these challenges, we introduce a method for performing microscale IP using magnetic particles and digital microfluidics (DMF-IP). The new method allows for 80% recovery of model proteins from approximately microliter volumes of serum in a sample-to-answer run time of approximately 25 min. Uniquely, analytes are eluted from these small samples in a format compatible with direct analysis by mass spectrometry. To extend the technique to be useful for large samples, we also developed a macro-to-microscale interface called preconcentration using liquid intake by paper (P-CLIP). This technique allows for efficient analysis of samples >100× larger than are typically processed on microfluidic devices. As described herein, DMF-IP and P-CLIP-DMF-IP are rapid, automated, and multiplexed methods that have the potential to reduce the time and effort required for IP sample preparations with applications in the fields of pharmacy, biomarker discovery, and protein biology.



Immunoprecipitation (IP) is a widely used technique in which target molecules in complex samples are captured by antibodies (or other capture molecules like Protein A or G) bound to insoluble supports. The target molecules are then eluted from the supports without background contaminants, greatly simplifying subsequent quantitation and analysis for applications including medical diagnostics and forensic sciences.[1,2] Importantly, in contrast to other kinds of preconcentration/sample cleanup techniques, IP elution conditions are designed to be gentle, such that target molecules maintain their structure and functionality throughout the process. This allows for capture, cleanup, and recovery while maintaining associated target-binding partners, e.g., an enzyme bound to an inhibitor can be captured on the insoluble support and the complex can be eluted intact.[3−5] IP has become increasingly prevalent as the pharmaceutical industry has pivoted to focus on the development of biologically derived drugs.[6−8] These applications typically involve complex matrices like blood and serum[9] or cell lysate,[10] and IP can effectively remove the background matrix, preparing the analytes for the analytical method of choice for this application, mass spectrometry (MS).

In its most common format, immunoprecipitation is implemented using magnetic particles with covalently attached antibodies. The process is typically tedious, requiring that a magnet be held adjacent to a vial containing the sample, such that the particles become immobilized, allowing supernatant to be removed and exchanged. Magnetic separation racks (e.g., the MagnaRack from ThermoFisher[11]) alleviate some of the tedium, and robotic systems (e.g., the Abnova Precipitor[12]) can automate some of the steps in settings for which cost is not a concern. However, all of these options are designed to operate with large volumes, typically 100 μL of sample or more. This requirement precludes the use of precious samples such as pin-pricks of blood or core-needle biopsies.

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The challenge described above has driven interest in miniaturizing IP using microfluidic devices relying on enclosed microchannels. This approach has been used in the analysis of peptides,[13] proteins,[14] and DNA[15−18] (the latter via chromatin immunoprecipitation). While these studies represent important steps forward, the methods do not lend themselves to straightforward integration for analysis by mass spectrometry, as they are limited by the complexities of integrating membranes and sonicators within devices,[13,16] MS-incompatible elution media,[14] or the necessity of protein elution off-chip into microcentrifuge tubes.[17,18] Furthermore, most of these studies also rely on external mechanical pumps which can be cumbersome to operate and automate for multiplexed analysis.

In an effort to address the challenges described above, we introduce a technique for performing IP using digital microfluidics (DMF). DMF is a microscale fluid handling technique that operates by manipulating nano- to microliter fluid droplets on an open array of insulated electrodes, by applying a programmed series of electrical potentials.[19] All of the operations are automated and computer-controlled, which greatly increases the reproducibility and allows parallel handling of multiple samples simultaneously. Many different types of fluids are compatible with DMF, including protein-rich biological samples and polar organic solvents, meaning that DMF is suitable for the reagents used in IP.[20] Most importantly, DMF is well suited for handling suspended magnetic particles[21] for applications such as heterogeneous immunoassays,[22−26] oligonucleotide binding assays,[27−29] and high abundant protein depletion.[30] Finally, because of the open nature of the DMF array, there is no risk of clogging (a common problem for applications involving precipitation in microchannels).

Here, we describe a method for immunoprecipitation by digital microfluidics (DMF-IP) for sample cleanup and targeted protein preconcentration. We demonstrate that DMF-IP is effective for purifying model protein analytes found in undiluted serum such that the analytes can be quantified by direct-elution mass spectrometry. Furthermore, to extend the technique to be useful for large samples, we developed a macro-to-microscale interface called preconcentration using liquid intake by paper (P-CLIP). To our knowledge, this report is the first to describe the integration of DMF with immunoprecipitation, and we propose that the new hyphenated technique will be useful for a wide range of applications.

EXPERIMENTAL SECTION

Reagents and Materials. Unless otherwise specified, reagents were purchased from Sigma-Aldrich (Oakville, ON), and deionized water (DI water) with a resistivity >18 MΩ·cm was used to prepare all aqueous solutions. An aqueous stock suspension of superparamagnetic particles with an antihuman serum albumin antibody coating (LSKMAGL02) was purchased from Millipore (Billerica, MA). Human serum albumin (HSA, molecular weight based on amino acid composition of 66 437 Da) solutions were prepared from lyophilized solids. Fluorescein-isothiocyanate labeled human serum albumin (FITC-HSA) was purchased from GenScript USA Inc. (Piscataway, NJ). All solutions used in DMF devices contained 0.1% Tetronic 90R4 (BASF Corp., Germany) surfactant additive.

Device Operation and Fabrication. Digital microfluidic devices comprised two plates and were assembled (as detailed elsewhere[30]) at the University of Toronto Nanofabrication Centre (TNFC). The bottom plates comprised a 15 × 4 array of square driving electrodes (2.2 × 2.2 mm each) and 12 large (16.4 × 6.7 mm) reservoir electrodes. These bottom plates were coated with ∼7 μm Parylene-C (Specialty Coating Systems, Indianapolis, IN) by vapor deposition before being spin coated with ∼200 nm Teflon-AF (DuPont, Wilmington, DE) and baked at 160 °C for 10 min. Top plates were 0.7 mm thick indium tin oxide coated glass substrates (Delta Technologies, Ltd., Loveland, CO). The top plates were also coated with ∼200 nm of Teflon-AF, but the 10 min baking was performed at 235 °C. Top plates were attached to bottom plates using double sided tape spacers with an interplate distance of ∼180 μm. This results in a unit droplet volume on a single actuation electrode of approximately 1 μL; larger volumes were handled by simultaneously actuating multiple electrodes.

DMF experiments were implemented using an automation system (described in detail elsewhere[23]) that features a magnet mounted on a step-motor which can be moved to enable separation of magnetic particles on the device. The automation system (including the magnet) was programmed and managed by Microdrop, an open-source application for the manipulation of droplets on DMF devices.[31] Droplets were driven by applying 100−120 V_{RMS} sine waves at 10 kHz between the top plate counter-electrode and successive driving electrodes on the bottom plate. All reagents were loaded onto the device from the edge of the top plate by actuating reservoir electrodes underneath the reagents. Extended droplet mixing was performed as described previously[23] to ensure rapid dispersion of magnetic particles.

DMF-IP General Procedure. Immunoprecipitation was performed in eight steps as depicted in Figure 1 with minor variations for specific applications (outlined in the following sections). (1) A suspension of magnetic particles was loaded to a reservoir, and then, the entire volume was driven onto the device (volume and density dependent on application). (2) The magnet was engaged, and the particles were immobilized to the device surface as the droplet was driven to a waste reservoir. (3) A 4 μL droplet of sample was loaded into a reservoir and driven onto the immobilized particles; the magnet was disengaged, and the droplet was mixed for 10 min to resuspend the particles. (4) Step (2) was repeated. (5) A 4 μL droplet of Tris-HCl buffer (pH 7.6, Tris-buffer) was loaded into a reservoir and driven onto the immobilized particles; the magnet was disengaged, and the droplet was mixed for ∼3 min, followed by a repeat of step (2). (6) Step (5) was repeated. (7) A 4 μL droplet of 100 mM citric acid was loaded onto a reservoir and driven onto the particles; the magnet was disengaged, and the droplet was mixed for 5 min (unless otherwise noted). (8) The magnet was engaged to separate the particles for a final time, and the resulting droplet was collected for analysis by disassembling the device.

DMF-IP Elution Efficiency. Elution efficiencies were evaluated using a sample comprising 200 μg/mL FITC-HSA in 80% fetal bovine serum (FBS) and 20% DI water. The protocol followed the eight steps outlined above, but with the following modifications. The stock suspension of magnetic particles was diluted 1/4 in DI water and was loaded as a 4 μL aliquot in step (1). Step (7) was performed with mixing durations of: 1, 2, 5, and 10 min, and all of the mixing steps were performed in the dark. After completing step (8), 1 μL of the eluted droplet was collected and diluted to 100 μL in Tris-buffer and vortexed briefly. Each diluted sample was loaded into

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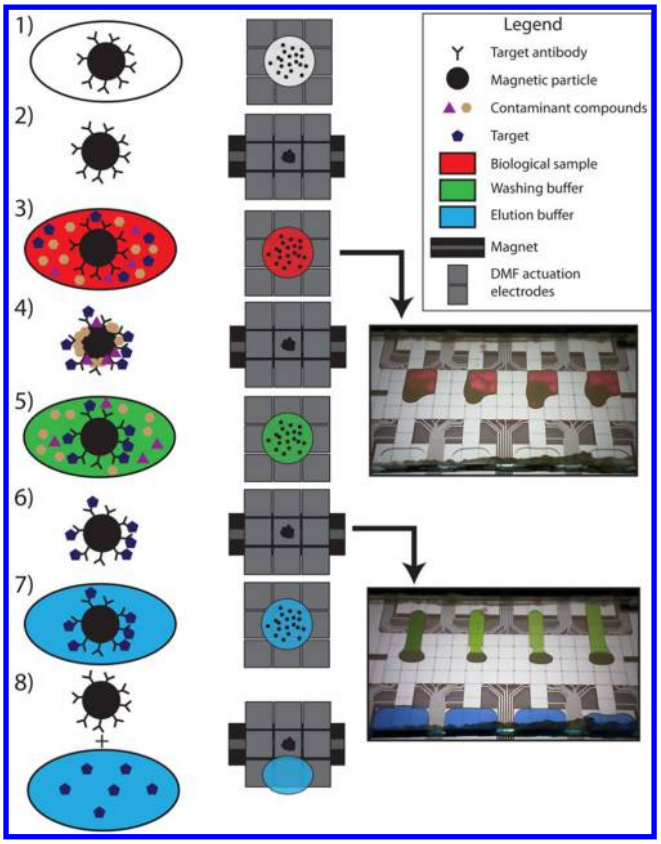

Figure 1. Immunoprecipitation by digital microfluidics (DMF-IP). Left: graphical representation of the eight-step DMF-IP procedure, focused on the surface of a single particle. Center: graphical representation of the eight-step procedure, focused on the droplets and particles on the DMF device. Right: photographs of the DMF device surface during DMF-IP at steps (3) and (6). Food coloring dyes were added to improve droplet visibility. Steps include: (1) Antibody-modified particles are loaded onto the device. (2) Particles are isolated. (3) Particles are exposed to the sample. (4) Particles are isolated. (5–6) Particles are washed and isolated. (7) Analytes are eluted. (8) Eluted analytes are recovered for analysis.

a 96 well plate, and the fluorescence intensity was measured with a plate reader (PHERAstar microplate reader, BMG Labtech, Ortenberg, Germany). Measurements were performed in "end point" mode (COSTAR 96 well plate geometry) with 10 flashes per well and a gain of 701 using 485 nm excitation and 520 nm emission filters. The FITC-HSA concentration in each eluted droplet was determined by comparing the measured fluorescence intensity to a calibration curve of standards (0, 10, 50, 100, and 200 μg/mL FITC-HSA in Tris-buffer) evaluated using the same conditions. Each calculated eluted concentration was referenced to the initial sample concentration to determine the elution efficiency for each elution time. Each condition was evaluated in triplicate using the same DMF device.

The elution efficiency for multiple elution steps was evaluated as described above but for a single mixing duration: 5 min. In these experiments, after step (8), the device was reassembled and steps (7) and (8) were repeated using a fresh aliquot of citric acid. This process was repeated twice more to collect droplets from one, two, three, and four elution steps. The collected samples were then measured, and the concentration was determined and compared to that of the initial droplet as above. Each condition was evaluated in triplicate using the same device.

Sample Clean-Up by DMF-IP. Immunoprecipitation was performed on FBS containing 1 mg/mL HSA using the eight-step protocol described above, with the following modifications. The stock suspension of magnetic particles was diluted 1/2 with DI water and then was loaded as a 5 μL aliquot in step (1). After step (8), 3 μL of the eluted droplet was collected and diluted to 5 μL with DI water and then processed for MS analysis as described below. Each condition was evaluated in triplicate using the same device.

DMF-IP with Preconcentration Using Liquid Intake by Paper (P-CLIP). DMF devices were formed as above, except triangular pieces cut from a KimWipe were placed onto some reservoirs such that a point penetrated into the array of actuation electrodes. Another piece of KimWipe was positioned adjacent to each triangle, such that they presented a contiguous network of paper. Samples of FBS containing 30 μg/mL HSA were prepared and treated by two methods. In the first method (with no preconcentration), the eight-step DMF-IP procedure described above (with a 4 μL aliquot of sample) was performed, using a 1/2 dilution of the stock suspension of magnetic particles in DI water (loaded as a 5 μL aliquot) in step (1). In the second method (powered by P-CLIP), a 100 μL aliquot of sample was combined off-chip with 2.5 μL of the stock suspension of magnetic particles in a microcentrifuge tube, which was then mixed for 1 h using a rotating vial rack. The 102.5 μL sample was then loaded onto a reservoir on a DMF device (adjacent to the edge of the top plate) where a modified DMF-IP procedure was performed in eight steps. (1A) A "tongue" of sample was pulled from the sample reservoir to the center of the device, and the magnet was engaged. (2A) The tongue was then driven to the opposite side of the device, such that it encountered the KimWipe. (3A) The wicking action of the KimWipe pulled the volume of sample across the device. (4A) The remaining sample was passively drawn into the wipe, leaving the magnetic particles immobilized on the center of the device. During this step, the electrodes remained actuated, defining the path for the sample to flow across the device. The electrodes were sequentially turned off (starting with the electrode adjacent to the source reservoir) as the final volume of solvent was pulled into the waste reservoir. (5A)–(8A) were identical to steps (5)–(8) above. After step (8A), 3 μL of the eluted droplet was collected and diluted to 5 μL with DI water and then processed for MS analysis as described below. Each condition was evaluated in triplicate using the same device.

Mass Spectrometry. Samples collected from DMF devices [as well as 5 μL control samples including (a) 1 mg/mL HSA in Tris-buffer containing 0.1% Tetronic 90R4 or (b) 1 mg/mL HSA in FBS containing 0.1% Tetronic 90R4] were diluted to 45 μL in a centrifuge tube with an aqueous solution of 9 M urea; the mixture was vortexed briefly and then allowed to incubate for 5 min at room temperature. Then, 2.25 μL of aqueous 100 mM tris(2-carboxyethyl)phospine (TCEP) was added; the mixture was vortexed briefly and then incubated for 30 min at room temperature. 4.7 μL of aqueous 100 mM chloroacetamide was added; the sample was vortexed briefly and incubated for 30 min at room temperature in the dark. The sample was diluted to 500 μL in aqueous 25 mM ammonium bicarbonate, and then, an aliquot of aqueous trypsin was added (containing 1.25 μg of trypsin for samples that originally contained 1 mg/mL HSA or 200 ng of trypsin for samples that originally contained 30 μg/mL HSA). The mixture was

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incubated at 37 °C for 4 h and then quenched by adding 7 μL of 5% formic acid. All samples were frozen at −80 °C until analysis.

Before analysis, each sample was thawed and subjected to solid-phase extraction using C18 solid phase extraction pipet tips (Pierce C18 Tips, 100 μL bed, #87784) as per the manufacturer's instructions. In brief, the tips were wetted in 50% acetonitrile (ACN) in DI water and then equilibrated in 0.1% trifluoroacetic acid (TFA) in DI water. The entire sample (above) was then iteratively aspirated into and dispensed from the tip for 10 cycles. The tips were then rinsed twice with 100 μL of 0.1% TFA, 5% ACN in DI water. Samples were then eluted in 100 μL of 0.1% acetic acid, 60% ACN in DI water. Finally, an internal standard of angiotensin I was added (final concentration of 5 μg/mL), and the sample was evaluated by direct injection mass spectrometry at a flow rate of 5 μL/min.

Samples were analyzed using a SCIEX API 4000 (SCIEX, Concord, ON) triple quadrupole mass spectrometer operating in tandem (MS/MS) mode. Samples were introduced via a small volume, 25 μm internal diameter electrospray ionization interface (SCIEX). The source temperature was 150 °C with an electrospray voltage of 5000 V. The collision gas, curtain gas, and sheath gas (GS1, GS2) pressures were 4, 10, 15, and 15 psi, respectively. Quadrupoles Q1 and Q3 were set at unit resolution. For HSA analysis, the precursor tryptic peptide fragment $LVNEVTEFAK^{2+}$ was isolated at m/z 575.3 using a declustering potential of 101 V and fragmented at a collision energy of 23 eV and a collision cell exit potential of 8 V. The HSA peptide product ion ($NEVTEFAK^{+}$) was isolated at m/z 937.6. The internal standard (I.S.) precursor ion (human angiotensin I, $DRVYIHPFHL^{3+}$) was isolated at m/z 433 using a declustering potential of 91 V and fragmented with a collision energy of 29 eV and a collision cell exit potential of 10 V. The I.S. product ion (histidine immonium ion fragment) was monitored at m/z 110. The dwell time for both species was 150 ms, and 12 scans were collected for each ion in both MS2 scanning (MS2 m/z range of 900−980 for the HSA tryptic fragment and 100−200 for the I.S.) and multiple reaction monitoring (MRM) modes. Peaks were viewed and measured using SCIEX PeakView v 1.2.2.0 and SCIEX Analyst v 1.6.2 software. For quantitative analysis of replicate samples, results were expressed as the ratio of peak heights of the analyte product ion to that of the I.S.

Conformation, Purity, and HPLC-MS. Analysis of protein conformation, the purity of samples after DMF-IP processing, and analysis by HPLC-MS are described in the Supporting Information.

RESULTS AND DISCUSSION

Digital Microfluidic Immunoprecipitation (DMF-IP). IP is a powerful technique for working with biological samples, particularly for purifying a specific target while maintaining the target's conformation and intermolecular associations, but it can be time-consuming and tedious and uses large volumes of samples. The overall goal of this work was to address the challenges of automating and miniaturizing IP using digital microfluidics and antibody-functionalized magnetic particles.

As shown in Figure 1, an eight-step procedure was developed to facilitate DMF-IP on sample volumes ranging from 3 to 5 μL. The left column shows a graphical representation of the surface of a magnetic particle during each step, and the central column shows a graphical representation of what is occurring on the DMF device. In step (1), magnetic particles are loaded into reservoirs and then driven onto the electrodes. In step (2), the magnet underneath the device is engaged to immobilize the particles on the device surface, while the suspending liquid is removed. In step (3) (depicted in the upper inset photograph), a sample (red) containing the target analyte (black pentagons) and other solutes (purple triangles and orange hexagons) is moved to the immobilized particles; the magnet is disengaged, and the particles are actively resuspended. In step (4), the particles are isolated from the sample via magnetic separation, and in steps (5−6) (depicted in the lower inset photograph), the particles are washed in washing buffer (green) twice. Then, in step (7), the particles are exposed to elution buffer (blue), and finally, in step (8), the particles are removed and the purified target is recovered. In practice, the device was capable of processing four samples simultaneously. The only manual steps required were the loading of sample and reagents and the removal of waste.

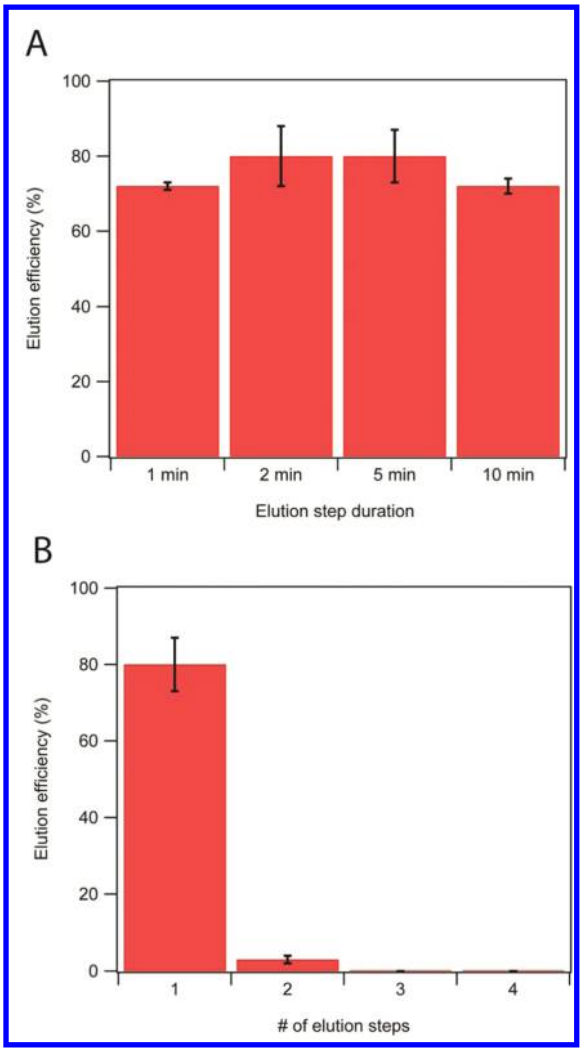

Figure 2. Optimization of DMF-IP elution efficiency. Samples comprised 4 μL aliquots of 200 μg/mL FITC-HSA in 80% fetal bovine serum. (A) Bar graph of elution efficiencies as a function of elution step duration. (B) Bar graph of elution efficiency as a function of the number of 5 min elution steps. Error bars represent ± 1 standard deviation.

Human serum albumin (HSA) was chosen as a model analyte, in recognition of its importance as a target for monitoring the metabolism of drugs or environmental toxins in

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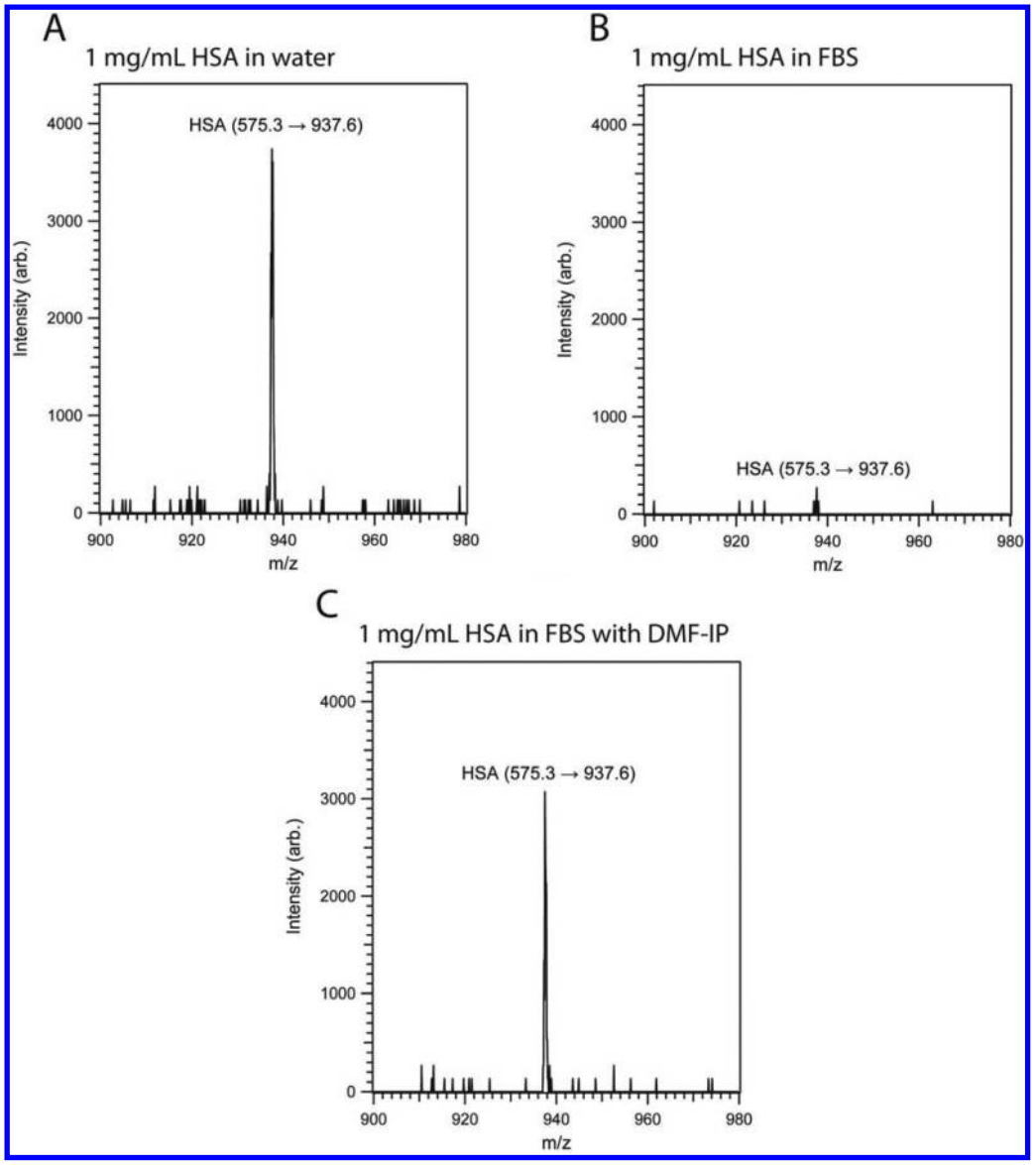

Figure 3. DMF-IP with analysis by mass spectrometry. Representative tandem mass spectra of a tryptic peptide of HSA (m/z 575.3 → m/z 937.6) prepared from 4 μL samples of 1 mg/mL HSA dissolved in (A) DI water (with no serum), (B) fetal bovine serum without DMF-IP, and (C) fetal bovine serum processed by DMF-IP.

pharmaceutical or clinical settings.[32] (For example, the presence of a mixed disulfide HSA adduct associated with environmental toxicants is often used to diagnose retardation of uterine growth in pregnant mothers.[33]) For all results described here, the sample comprised HSA dissolved (at various concentrations) in bovine serum. A number of different elution conditions were evaluated, eventually settling on an elution buffer of 100 mM citric acid. As described in the Supporting Information (and shown in Figure S1), this buffer was found to not (permanently) alter the conformation of the analyte (a key trait of good IP methodology). An assay was developed relying on fluorescently labeled HSA (FITC-HSA) to determine (a) the optimum elution step duration and (b) the optimum number of elution steps required to maximize the recovered protein. For the former, it was determined that a 1 min elution step facilitates recovery of 72 ± 1% (average ±1 std. dev.) of the analyte (Figure 2A). Increasing the elution step duration to 2, 5, and 10 min yielded recoveries of 80 ± 8%, 80 ± 7%, and 72 ± 2% with an overall mean recovery of 76 ± 5% across all time points. The differences between these recoveries were not significant (single factor ANOVA, α = 0.05, p = 0.170); thus, we arbitrarily chose to use 5 min elution steps for the remainder of the work described here. For the latter experiment, as shown in Figure 2B, a single 5 min elution step recovered 80 ± 7% of analyte, and a second step recovered an additional 3 ± 1%. Third and fourth treatments did not yield detectable amounts of analyte.

In practice, it was decided that a single 5 min elution step (with 80% analyte recovery) was sufficient for the work described here, which resulted in a run time (from sample loading to analyte recovery) of 25 min. The purity of the analyte eluted under these conditions was also assessed by protein gel electrophoresis (Figure S2) relative to the wash droplets and depleted serum after IP. Note that 80% recovery is consistent with or higher than the recoveries reported for conventional[34,35] and microfluidic[13,14,17,18] IP protocols, with the caveat that recoveries depend on antibody−antigen binding kinetics (making comparisons between different systems

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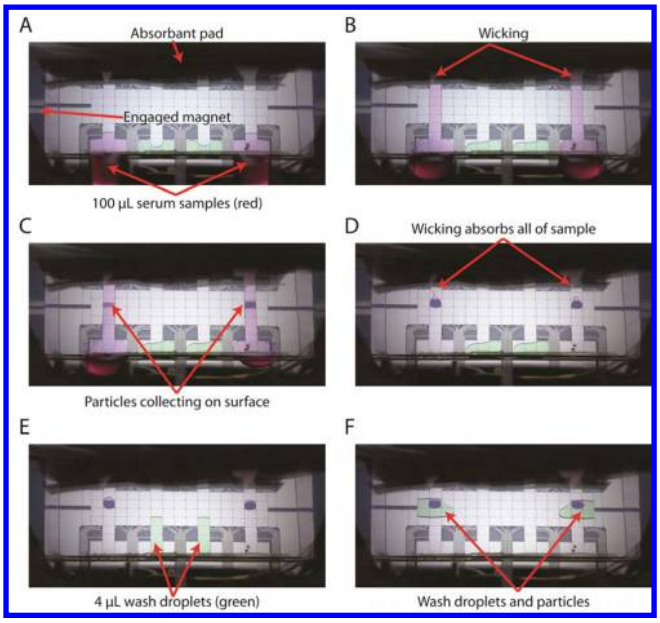

Figure 4. Preconcentration of magnetic particles using liquid intake by paper (P-CLIP). Frames from a movie showing (A) initial device setup featuring two 100 μL serum samples (red, bottom) and absorbant pads (top); (B) "tongues" of fluid from the samples driven by DMF actuation over the engaged magnet; (C) particles collecting onto the device surface as the fluid wicks into the absorbant pad; (D) the sample nearly completely absorbed, leaving a small volume (and bolus of particles) behind; (E) wash droplets (green) moving toward the particles; (F) resuspension of preconcentrated particles into wash droplets. Food coloring dyes added to improve visibility.

problematic). Most importantly, the ability of the new method reported here to operate on 3−5 μL volumes in a format convenient for mass spectrometry should potentially allow seamless integration with analysis of precious samples such as dried blood spots[36,37] or core-needle biopsies.[38,39]

The gold standard analytical technique used for biologically derived drug analysis is mass spectrometry (MS). To evaluate the compatibility of this technique with MS, fetal bovine serum samples containing 1 mg/mL HSA were analyzed before and after performing DMF-IP. Figure 3 shows representative ESI-MS-MS spectra of three types of samples (A) HSA in DI water, (B) HSA in bovine serum (not subjected to DMF-IP), and (C) HSA in bovine serum which was subjected to DMF-IP. In each analysis, samples were processed and MS-MS transitions were monitored from a precursor ion (the tryptic peptide of HSA, $LVNEVTEFAK^{2+}$, m/z 575.3) to all product ions in the m/z range of 900−980. As shown in a representative spectrum of the standard in water (Figure 3A), a strong signal from the y8 product ion ($NEVTEFAK^{+}$) is observed at m/z 937.6 with very little background signal. In contrast, in a representative spectrum of the standard in serum with no IP (Figure 3B), there is low signal at the designated transition. Finally, in a representative spectrum of the standard in serum processed by DMF-IP (Figure 3C), the y8 product ion signal is high. This highlights the need for IP; even when using an exquisitely selective technique like MS-MS (targeting a known analyte's transition), IP-pulldown is required for robust detection. Specifically, in this case, when using an internal standard (to compensate for variations in ionization efficiency), the DMF-IP method (Figure 3C) rescues a signal that is nearly undetectable in the control (Figure 3B), improving the signal by a factor of

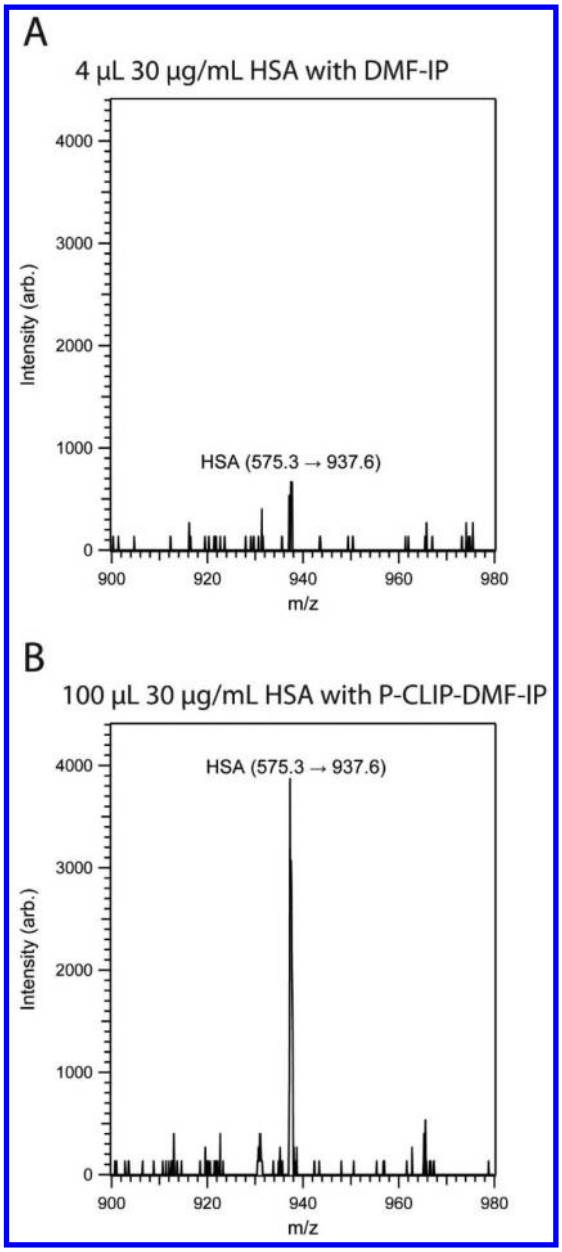

Figure 5. Representative tandem mass spectra of a tryptic peptide of HSA (m/z 575.3 → m/z 937.6) prepared from 30 μg/mL HSA in serum, analyzed from (A) a 4 μL sample processed by DMF-IP with no preconcentration (as in Figure 1) or (B) a 100 μL sample preconcentrated and processed by P-CLIP-DMF-IP (as in Figure 4).

3.7 (the ratio of HSA product ion intensity/internal standard product ion intensity for the DMF-IP sample divided by the same ratio for the untreated sample) while maintaining an acceptable level of variance on the DMF-IP recovered samples of 7% relative standard deviation (RSD, n = 4).

For the data shown in Figure 3, the samples were processed by IP on-chip (as per Figure 1), but the processing steps of reduction, alkylation, and digestion, as well as solvent-exchange by solid-phase extraction (SPE) were carried out off-chip. In the future, it should be straightforward to integrate the latter steps on-chip as well (as reported previously for DMF-proteomic processing[40−42] and DMF-SPE[43−45]), such that the DMF device generates fully processed analyte (i.e., tryptic peptides in MS-compatible solvent from proteins captured by

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IP) directly from undiluted serum. Note that the detection method in this work was direct-infusion MS/MS; in the future, it should also be possible to integrate the method described here with an automated DMF-HPLC-MS interface (as reported previously[46]). This would improve the sensitivity of the technique and would provide a complete sample-to-answer solution appropriate for a wide range of applications in biologic pharmaceutical analysis.[6−8] A preliminary investigation (see Figure S3) combining DMF-IP with HPLC-MS/MS shows effective detection of HSA at concentrations as low as 50 ng/mL in serum.

DMF-IP with Preconcentration Using Liquid Intake by Paper. As described above, the capacity to work with small-volume samples is a unique advantage of the technique described here. However, this advantage can also be a limitation; when not working with a precious sample, it often is desirable to be able to analyze a large volume, allowing the observation of analytes present at very low concentration. This limitation is a critical drawback for DMF and most other microscale analysis techniques. To overcome this limitation, we developed a new macro-to-microscale interface that integrates preconcentration with DMF-IP, allowing for the evaluation of much larger sample sizes.

In developing the new method, we considered a variety of formats. For example, one mechanism to evaluate a 100 μL sample volume would be to dispense (for example) twenty-five 4 μL droplets of sample onto the electrode array, process them in parallel or in series, and then pool the final eluents together for analysis. This idea was discarded as being unwieldy and slow; we wanted to develop a much simpler and faster method for straightforward operation. Our eventual solution[47] relies on (a) premixing a large-volume sample (in this case,100 μL) with magnetic particles (off-chip), followed by (b) isolation and resuspension of the particles on-chip in a small-volume droplet (in this case, 4 μL) for further processing. We call the new technique "preconcentration using liquid intake by paper" (P-CLIP).

The new P-CLIP method is depicted in Figure 4. A key feature is the incorporation of lateral-flow pumping[48,49] to facilitate rapid and passive handling of the large fluid volume. As shown, a large volume of sample (premixed with magnetic particles) is loaded onto the device, and then, a portion of the sample is driven across the array while the magnet is engaged (Figure 4A). Upon reaching the other side of the device, the sample contacts an immobilized paper pad, which continuously wicks the sample across the device (Figure 4B). Because the magnet is engaged, the magnetic particles become immobilized over the top of the magnet on the device surface (Figure 4C). Finally, a combination of wicking and DMF actuation removes the remainder of the sample from the magnetic particles (Figure 4D), and the standard DMF-IP protocol can then be implemented as normal in small droplet volumes (Figure 4E,F). We have demonstrated the compatibility of P-CLIP with immunoassays under various conditions;[47] here, we report its application to DMF-IP.

To test the effectiveness of P-CLIP for the analysis of dilute analytes, FBS samples were spiked with 30 μg/mL HSA and analyzed using DMF-IP (Figure 1) and P-CLIP-DMF-IP (Figure 4) using direct injection mass spectrometry. Representative spectra are shown in Figure 5. As shown, a small volume of sample (i.e., with no preconcentration by P-CLIP) produces a product ion peak that has low signal-to-noise (Figure 5A). While this type of sample might be easily detected if evaluated with HPLC-MS/MS and/or with a newer spectrometer (Figure S3), there simply is not enough analyte to produce a usable signal in the system used here. In contrast, when 100 μL of the same sample was concentrated to 4 μL using P-CLIP, the HSA product ion peak has high intensity (Figure 5B), representing a signal increase of 12 times (with 14% RSD on P-CLIP samples, n = 4) relative to the measurement without P-CLIP. The increased variance for this technique is likely a function of variations in bead-retrieval efficiencies during the concentration step (a topic of ongoing study[47]). Finally, in this work, 100 μL samples were processed two-at-a-time; in the future, slight device modifications should allow for many more preconcentration operations to be conducted in parallel on a single device. More work is needed, but we propose that P-CLIP is an attractive solution to the long-standing problem of limited sample sizes in DMF.

CONCLUSION

We have developed the first digital microfluidic method for performing immunoprecipitation. The method was applied to analyzing human serum albumin in serum samples using digital microfluidic manipulation of antibody-functionalized magnetic particles with and without preconcentration. We believe this technique has great potential for the analysis of proteins in the fields of pharmacy and medicine including elucidation of protein structure and associated molecules.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b02915.

HSA conformation experiments; gel electrophoresis experiments; HPLC-MS experiments; effects of immunoprecipitation-elution buffer on protein conformation; Coomasie Blue-stained SDS-PAGE gel; HPLC-MS/MS chromatogram (PDF)

AUTHOR INFORMATION

Corresponding Author

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

Notes

The authors declare no competing financial interest.

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